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THE UNIVERSITY OF ALBERTA

ALTERATIONS IN PHOSPHOLIPID METABOLISM IN PERFUSED RABBIT CAROTID ARTERY IN RESPONSE TO DE-ENDOTHELIALIZATION

by

Gary A. Simatos

©

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF PHYSIOLOGY

EDMONTON, ALBERTA

FALL 1987

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ISBN 0-315-41038-8

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TITLE OF THESIS: ALTERATIONS IN PHOSPHOLIPID METABOLISM IN PERFUSED RABBIT CAROTID ARTERY IN RESPONSE TO DE-ENDOTHELIALIZATION

DEGREE FOR WHICH THESIS WAS PRESENTED: DOCTOR OF PHILOSOPHY

YEAR THIS DEGREE GRANTED: FALL 1987

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled ALTERATIONS IN PHOSPHOLIPID METABOLISM IN PERFUSED RABBIT CAROTID ARTERY IN RESPONSE TO DE-ENDOTHELIALIZATION submitted by Gary A. Simatos in partial fulfilment of the requirements for the degree of Doctor of Philosopy.

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Supervisor

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Supervisor

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External Examiner

Date 15 Sept., 1983

DEDICATION

To Bobby, Mike, Susie, Vicky and Grand Mere in Montreal.

I've missed being with you these past six years.

ABSTRACT

Incorporation of ^{3}H-acetate into neutral and polar lipids was studied in perfused rabbit carotid artery in the presence and absence of an intact endothelium. Scanning electron miscroscopy was used to confirm the condition of the endothelium during the experimental period. Arteries were maintained under pressure throughout. In intact arteries, prolonged perfusion with lipoprotein deficient serum (LPDS) instead of complete fetal calf serum (FCS), induced an increase of ^{3}H-acetate incorporation into cholesterol, suggesting that the regulation of cholesterol biosynthesis by serum lipoproteins persists in this tissue under these conditions. In 16 hour perfusions with endothelium removed, whole serum or lipoprotein deficient serum caused a two- to four-fold increase of ^{3}H-acetate incorporation into phospholipid, most notably phosphatidylcholine (PC) with no obvious change in cholesterol synthesis. Lipid determination showed essentially the same amounts of PC phosphorous present in both intact and de-endothelialized arteries suggesting greater PC turnover and not increased net PC synthesis. Similar experiments were run using ^{14}C-choline in addition to ^{3}H-acetate. Incorporation of ^{3}H-acetate into PC in de-endothelialized arteries was again found to be two to four times that of PC in control arteries while ^{14}C-choline incorporation remained the same in the two groups. Hydrolysis of PC from de-endothelialized segments with phospholipase A2 (PA2), liberated sn-2 fatty acyl chains with specific activities two to four times that of control. Residual lyso PC from control and de-endothelialized segments was found to have similar specific activities between the two

groups. Fatty acid composition of complete PC, lyso PC and sn-2 fatty acid were evaluated by gas chromatography which showed no preferential deacylation or reacylation. These results suggest that de-endothelialization promotes an increase in fatty acid turnover at the sn-2 position of the PC molecule and that this process does not appear to involve preferential incorporation or release of a particular fatty acid species. These observations are seen only in the presence of serum supplemented perfusate (FCS or LPDS) and are abolished when serum-free media was used. Precipitation of LPDS with ammonium sulphate also abolished this effect suggesting that the serum factor responsible is a protein.

ACKNOWLEDGEMENTS

I am pleased to recognize the many friendships formed during my stay in the Department of Physiology.

Special thanks to Drs. Ken Hutchison and Mark Poznansky for support and encouragement. Thanks also to Deanna Moores, Donna Taylor and Sandra Czekanski for their good humour and making the lab an enjoyable place to work.

Thanks especially to Drs. Phil Davis, P. Thomas and Rajan George for many stimulating discussions and friendship. Also, thanks to my fellow grad students, Joy Steele, Don Robertson and Glen Wheeler who were there at the first and more recently Ian Gynther and John Greer.

Finally my sincerest appreciation to Annette Aarbo, Nick Diakiw, Ed Karpinski, Greg Borne, Joy Steele and last but not least Wendy Pearson for unfailing encouragement, support and friendship.

TABLE OF CONTENTS

Chapter Page

1. INTRODUCTION

1.1 General Introduction....1
1.1.1 Atherosclerosis: Description and Distribution....1

1.2 Anatomy and Biochemistry of the Artery....4
1.2.1 Introduction: Cells of the Artery Wall....5
1.2.1.1 The Endothelial Cell....5
1.2.1.2 The Smooth Muscle Cell....9
1.2.2 Blood Borne Cells....10
1.2.2.1 The Platelet....10
1.2.2.2 The Macrophage....11

1.3 Cholesterol and Atherogenesis....14
1.3.1 Introduction....14
1.3.1.1 Receptor Mediated Regulation of Cholesterol....16

1.4 Role of Phospholipids....19
1.4.1 Introduction....19
1.4.1.1 Phospholipids in Membranes....19
1.4.1.2 Phospholipid Synthesis and Regulation....20
1.4.2 Biologically Active Lipids....22
1.4.2.1 Arachidonic Acid Cascade....22
1.4.2.2 Phosphatidylinositol Cycle....24
1.4.3 Effect of Membrane Fluidity....25

1.5 Phospholipases....27
1.5.1 Introduction....27
1.5.2 Phospholipase Species....27

1.6 Fatty Acid Turnover....30

1.7 Theories of Atherogenesis....31

1.8 Endothelial Removal and Compositional Changes to The Artery....33
1.8.1 Problems and Experimental Approaches....33

1.9 Objectives....37

2. MATERIALS AND METHODS.....38

2.1 Materials.....38

2.2 Description of perfusion apparatus.....39

2.3 Surgical removal of carotid artery segments under pressure.....44

2.4 Perfusate preparation.....48

2.5 Scanning electron microscopy.....49

2.6 Tissue analysis.....50
2.6.1 Gas chromatography.....51
2.6.2 Hydrolysis of phosphatidylcholine by phospholipase A2.....51

2.7 Experimental design.....53
2.7.1 Tissue incubation.....53
2.7.2 Cholesterol biosynthesis in perfused arteries.....53
2.7.3 Effect of de-endothelialization.....54
2.7.4 Movement of ^{3}H-acetate across the artery wall.....54
2.7.5 Effect of serum concentrations.....55
2.7.6 Short term perfusion.....55
2.7.7 Demonstration of PC turnover: label before removing endothelium.....55
2.7.8 Double label: ^{3}H-acetate and ^{14}C-choline.....56
2.7.9 Bioassay of LPDS subfractions.....56
2.7.10 Effect of endothelial conditioned media on PC turnover.....56

2.8 Statistical analysis.....57

3. RESULTS.....58

3.1 Tissue Incubations.....58
3.1.1 Cholesterol biosynthesis in minced arterial tissue....58
3.1.2 Incubation of tissues with or without endothelium.....60

3.2 Experimental Protocol.....62
3.2.1 Time course.....62

3.3 Perfusion Experiments.....63
3.3.1 Scanning Electron Microscopy.....63
3.3.1.1 Verification of Intact Endothelium.....63

3.3.2 Cholesterol biosynthesis in Perfused Arteries with Endothelium using Fetal Calf Serum or Lipoprotein Deficient Serum.....68

3.4 Perfusion Experiments on Segments With or Without Endothelium...71
3.4.1 Perfusion With Fetal Calf Serum.....71
3.4.2 Effects of Lipoprotein Deficient Perfusate.....73
3.4.3 Effect of Serum-Free Perfusate.....75
3.4.4 Effect of Increasing Concentration of Lipoprotein Deficient Serum in De-endothelialized Arterial Segments.....75
3.4.5 Serum Factor: Ammonium Sulphate Fractionation.....78
3.4.5.1 Evaluation of Serum Sub-Fractions to Promote Phosphatidylcholine Acly Chain Turnover in De-endothelialized Arteries....78
3.4.5.2 SDS-Polyacrylamide Gel Electrophoresis.....81

3.5 Barrier Experiments.....83
3.5.1 Effect of De-endothelialization on ^{3}H-Acetate Flux Across the Artery Wall.....83
3.5.1.2 Movement of ^{3}H-Acetate from the Artery lumen into the Bathing Medium.....84
3.5.1.3 Movement of ^{3}H-Acetate from the Bathing Medium into the Perfusate.....86

3.6 Time Interval Experiments.....89
3.6.1 0-2 Hour Perfusion
0-4 Hour Perfusion
0-8 Hour Perfusion
0-12 Hour Perfusion

3.7 Phospholipid mass determination.....91

3.8 Pulse-Chase Experiments.....93
3.8.1 Preincubation with ^{3}H-Acetate Before De-endothelialization.....93
3.8.1.1 Analysis of Perfusate for Labelled Product.....94

3.9 Demonstration of Greater Fatty Acid Turnover in Phosphatidylcholine in De-endothelialized Arteries.....96
3.9.1 Double Label Perfusion Using ^{14}C-Acetate and ^{3}H-choline.....96

3.10 Phospholipid Analysis: Fatty Acid Composition.....99
3.10.1 Phospholipase A2 Hydrolysis of Phosphatidylcholine.....99
3.10.11 Gas Chromatography of Fatty Acid Composition Released sn-2 Fatty Acid and Residual Lysophosphatidylcholine.....101
3.10.12 Determination of ^{3}H-Acetate Incorporation into Released Fatty Acid and Residual Lysophosphatidylcholine.....105

3.11 Hybrid Experiment
3.11.1 Effect of Endothelial Cell Conditioned Media on Acyl Chain Turnover of Phosphatidylcholine in De-endothelialized Arteries.....107

4. DISCUSSION.....110

5. REFERENCES.....119

LIST OF TABLES

Table	Description	Page
1	Incorporation of ^{3}H-acetate into neutral and polar lipid in minced arterial tissue incubated for 16 hours in either 10% FCS or 10% LPDS in MEM.	59
2	Incorporation of ^{3}H-acetate into neutral and polar polar lipid in minced tissue with or without endothelium	61
3	Perfusion of arterial segments with 10% FCS or 10% LPDS in MEM	70
4	Ipcorporation of ^{3}H-acetate into lipid and ^{35}S-methionine into protein in arteries with or without endothelium perfused with 10% FCS	72
5	Ipcorporation of ^{3}H-acetate into lipid and ^{35}S-methionine into protein in arteries with or without endothelium perfused with 10% LPDS	74
6	Ipcorporation of ^{3}H-acetate into lipid and ^{35}S-mentionine into protein in arteries with or without endothelium perfused with serum-free media	76
7	Protein content of LPDS subfractions	79
8	Bioassay of LPDS ammonium sulphate precipitated subfractions	80
9	Bioassay of protein precipitated from LPDS	82
10	Incorporation of ^{3}H-acetate into phosphatidyl-choline in arterial segments perfused with 10% LPDS with or without endothelium for four time intervals	90
11	Determination of mass and specific activity of major phospholipid species	92
12	Total fatty acid composition of phosphatidylcholie from arterial segments with or without endothelium perfused with 10% LPDS in MEM	100
13	Hydrolysis of ^{3}H-DPPC with phospholipase A2	102
14	Fatty acid composition of lysoPC and sn-2 fatty acid of PC	104

15	^{3}H-acetate incorporation in lysoPC and sn-2 fatty acid from PC hydrolysed with phospholipase A2	106
16	Effect of endothelial cell conditioned perfusate on PC incorporation of ^{3}H-acetate in de-endothelialized arterial segments	109

LIST OF FIGURES

Figure	Description	Page
1	Cut-away section of the artery wall	5
2	Cleavage sites of the phospholipases	29
3	Perfusion apparatus	40
4	Detail of perfusion reservoir	41
5	Detail of tissue holder	43
6	Effect of increasing serum concentration on ^{3}H-acetate incorporation into PC	77
7	Monitoring of tissue bathing medium for the appearence of ^{3}H-acetate traversing the artery wall in perfused segments with or without endothelium	85
8	Monitoring of tissue perfusate for the appearence of ^{3}H-acetate crossing the artery wall in perfused segments with or without endothelium	87
9	Demonstration of increased turnover of PC in de-endothelialized arterial segments	95
10	Incorporation of ^{3}H-choline and ^{14}C-acetate into PC in perfused arterial segments with or without endothelium	98

LIST OF PLATES

Plate	Description	page
1	Placement of isolated arterial segment into tissue holder	46
2a-2d	Scanning electron micrographs of perfused rabbit carotid artery segments	64-67

LIST OF ABBREVIATIONS

AA	arachidonic acid
DG	diglyceride
DPPC	dipalmitoyl phosphatidylcholine
EC	endothelial cell
FCS	fetal calf serum
GAG	glycosaminoglycan
GC	gas chromatography
HDL	high density lipoprotein
HMG-CoA Reductase	3-Hydroxy-3-methylglutaryl coenzyme A
IDL	intermediate density lipoprotein
LDL	low density lipoprotein
LPDS	lipoprotein-deficient serum
MEM	minimum essential medium
PA2	phospholipase A2
PC	phosphatidylcholine
PDGF	platelet-derived growth factor
PE	phosphatidylethanolamine
PGI_2	prostacyclin
PI	phosphatidylinositol
PS	phosphatidylserine
Sil G	silica gel G
SM	sphingomyelin
SMC	smooth muscle cell
TLC	thin layer chromatography
VLDL	very low density lipoprotein

1. INTRODUCTION

1.1 General Introduction

1.1.1 Atherosclerosis: Description and Distribution

Cardiovascular disease (CVD) is the chief cause of morbidity and mortality in the western world. A characteristic exhibited by this disease, defying adequate explanation, is its predisposition to develop at certain specific sites in the arterial tree. The resulting patterns of distribution have been classified into broad categories (DeBakey, 1978)

The first of these patterns is disease that involves the major branches of the ascending aorta. From the point of view of disability and death, this form with its involvement of the coronary artery bed represents the most common and serious pattern. Plaque distribution may occur most severely in proximal or distal portions or affect the entire arterial bed.

A second category of plaque development involves the major branches of the aortic arch and includes the innominate, carotid and subclavian arteries and their major branches. Within this category several distribution patterns have also been observed. In over 50% of patients two or more arteries are affected with plaque being discretly localized in proximal regions of the vessels and thus surgical intervention is possible. Well localized lesions may also occur at the carotid bifurcations.

Both distal and proximal to these locations, the arteries appear to be relatively normal. Another pattern involves plaque in the distal arterial bed or intracranial portion, showing little disease of the proximal segments. This situation is not readily correctable by surgical treatment.

A third major pattern of plaque development occurs in the visceral branches of the abdominal aorta which includes the celiac axis, saphenous, mesenteric and renal arteries. This form also tends to be well localized occuring predominantly near the origin of these arteries with the aorta and leaving their distal regions largely unaffected.

A final anatomically distinct pattern involves the terminal abdominal aorta and its major branches. Characteristic development here includes disease at the aorto-iliac segment beginning near the origin of the iliac arteries and in the complicated situation, spreading to the origin of the renal arteries. Regions distal to those described are generally unaffected. Consequently this form of plaque can be corrected by surgical intervention.

The rate of plaque development has also been observed to fall into three general categories. The first is characterized by rapid development of stenosis or even occlusion from one to three years after angiographic evidence of initiation. Often such plaques are well localized and occur more commonly in the carotid, coronary and femoral arterial beds.

A more moderate rate of development is seen in a second group where clinical manifestations may take eight years from the first point of angiographic demonstration. Plaques in this group do occur with equal frequency throughout.

A third group represents the slowest to develop, in excess of ten years and in some patients, no progression is detectable 20 years after surgical correction.

The diverse distribution and variable rates of proliferation would suggest that the lesions generally attributed to atherosclerosis may represent the end result of a variety of pathologically discrete processes. It is clear that lesions vary considerably both in terms of their composition and preferred location in the arterial tree even though most forms ultimately share the commom result of occluding the arterial lumen , compromising circulation. An appreciation that plaque development may arise by several mechanistically independant processes may remove some of the confusion regarding the etiology of this disease.

1.2 Introduction

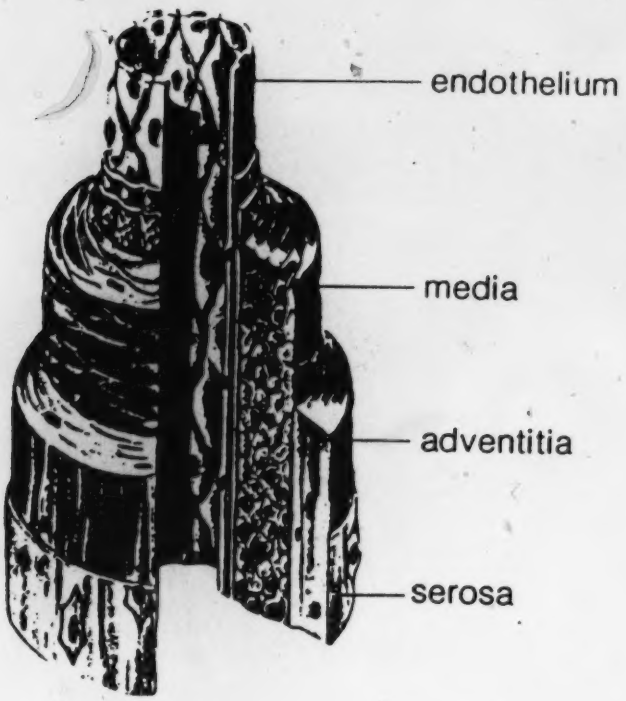
The Artery Wall: Anatomical and Biochemical Aspects

In dicussing atherosclerosis, it is important to bear in mind that the artery exists in a unique microenvironment. The pressurized interior contains blood which in addition to moving nutrients and waste, exerts a variety of hemodynamic forces within the artery. Such forces are dependant to a large extent on the local geometry of the vessel in question (Wesolowski et al, 1964, Ku et al, 1985).

Of the three layers making up the artery wall, the first inner-most layer which makes direct contact with the luminal contents is referred to as the intima. The intima consists primarily of an internal elastic lamina and the endothelium arranged in a monolayer lining the lumen. Though representing a small percentage of the total arterial tissue, the endothelial cell has been shown to mediate a host of routine functions and has been implicated to play a role in atherogenesis (for a review see Ross, 1986).

An intermediate region of smooth muscle cells (SMC) external to the intima and separated by an internal elastic lamina is that region referred to as the media. The external border of the media is also bound by a lamina, the external elastic lamina which delineates the border with the final external region of the artery, the adventitia, also made up primarily of SMC and connective tissue elements. The structural detail described is representative for the aorta while other arteries do show slightly different morphologies. A cross-sectional diagram of the artery wall is presented in Figure 1.

FIGURE 1. Cut-Away Section of the Artery Wall.



1.2.1 Cells of the Artery Wall

1.2.1.1 The Endothelial Cell

The endothelial cells are those cells that line the artery wall of all the vessels. They are arranged in a continuous monolayer and are contact inhibited. An important function of the endothelium is to provide a non-thrombogenic surface, that is a container that is unreactive with all blood cells. The endothelial cells of the large vessels form a tight impermeabile barrier to many proteins contained in blood (Huttner et al 1973). Morphologically, endothelial cells appear as thin elongated cells that form a variety of junctional complexes with adjacent cells (Simonescu et al, 1976). The borders between endothelial cells are said to be argyrophillic due to the unusual quality of these margins to take up silver salts. A unique feature to the endothelial cell is the presence of the Weibel-Palade body, an organelle found in no other cell type (Weibel and Palade,1964).

Endothelial cells are capable of producing and secreting a variety of substances (for a review see Ross, 1986). These include matrix proteins (Sage et al, 1979), prostacyclin, the most powerful inhibitor of platlet aggregation (Moncada, 1985), plasminogen activator (Levin and Loskutoff, 1978), thrombospondin (Jaffe and Mosher, 1978), and factor XIII antigen referred to as von Willebrand's factor (Caldwell, 1976) and a growth factor similar to platelet derived growth factor.

In terms of their relationship to other cells in the immediate vicinity, endothelial cells have been shown to produce vasoactive substances acting on arterial SMCs. Of interest is the observation that vasodilation in response to ACh occurs only in arterial preparations with an intact endothelium. This response is mediated by endothelium

derived relaxation factor (EDRF) described by Furchgott (1980). The identification of EDRF proved difficult due to its laibile nature and extremely short half-life. It was proposed that EDRF may be nitric oxide (NO) based on the observation that synthetic nitrovasodilators promoted responses similar to that elicited by EDRF. Recently, this was confirmed by Palmer (1987) who showed that NO is produced by endothelial cells and functions in a manner indistinguishable from EDRF. Endothelium may also interact with blood-borne components. In vitro studies using endothelial cell monolayers co-cultured with LDL produces a modified form of lipoprotein that is avidly taken up by macrophage cells (Henriksen et al, 1983).

A theory to explain the process of plaque development in the artery wall has been the suggestion that an early event is endothelial injury (Ross and Glomsett, 1976). Since endothelial cells can be readily grown in culture, many investigators have taken the in vitro approach to study these cells. The use of tissue culture has led to many remarkable strides in our understanding of cellular physiology and metabolism and a classic series using fibroblasts will be discussed below. Unfortunately some cell types grown in vitro show changes totally uncharacteristic of their in vivo counterparts. This has been shown to be the case for endothelial cells. Endothelial cells in vivo are largely quiescent while those grown in culture have a definite rate of turnover (Schwartz and Benditt, 1976). It has been proposed that enhanced turnover may more closely resemble "injured" or replicating cells in vivo. Therefore, the use of cultured ECs as a model of ECs in vivo may have limitations (Hanson and Schwartz, 1983). Consequently, many investigators have used an in vivo approach to answer questions

concerning endothelial cells. Of particular interest has been the examination of regions of endothelial regeneration. Using a variety of procedures, endothelium is removed and its mode of repair and replacement evaluated. It has been observed that endothelial cells will regenerate predominantly in a direction parallel to blood flow (Schwartz et al, 1978). Cells up to 100 cells away from the site of injury enter the "S" phase even though in vitro studies would suggest that such cells should be contact inhibited. In long term studies, Richardson (1980) showed that beneath regions of endothelial regeneration lipid accumulation will occur as well as increased content of glycosaminoglycans (GAGs). In vitro, Iverius (1972) showed that GAGs may form complexes with LDL suggesting a means whereby these complexes may curtail the normal flux of LDL in the artery wall.

The hemodynamic stresses that occur in the arterial tree are largely a function of the arterial geometry. In many regions, blood flow may deviate from what might be considered normal, laminar movement and generate isolated zones of flow disturbance (Wesolowski et al, 1962). It has been shown, again in vivo, that endothelial cells will alter their morphology and cytoskeletal elements in response to zones of high shear (Wong and Gotleib, 1984). Though the situation is far from clear cut, current understanding suggests that regions of high shear tend to protect endothelium while zones of low flow velocity accentuate plaque development (Zarins et al, 1983).

1.2.1.2 Smooth Muscle Cells

Smooth muscle cells make up the bulk of the tissue in the typical artery. With the arterial lumen lined by healthy endothelial cells SMC remain relatively quiescent. On their plasma membrane, these cells bear a variety of receptors including those that bind LDL (Chait et al, 1980) and platlet derived growth factor (Bowen-Pope and Ross, 1982). SMC themselves also produce a growth factor similar to platelet derived growth factor (Seifert et al, 1984). The presence of SMCs in the fatty streaks or fibrous plaques is well known. Thus there has been keen interest in studying the proliferative potential of SMCs. Work by Pietila and Nikkari (1983) showed that cultured SMC derived from atherosclerotic rabbits incorporate [3H]-thymidine to an extent greater than that observed in controls and that this increase persists in daughter cells. Benditt and Benditt (1973) suggested that SMC in the atheromateous plaque may originate from a single stem cell and proposed the monoclonal hypothesis for plaque development.

SMCs appear to be responsive to a host of chemoattractants and during their migratory or prolific phase produce large amounts of extracellular matrix material (Burk and Ross, 1979). In vitro studies have demonstrated that SMC can be induced to take up lipids and assume a foam cell-like appearance (Wolfbauer et al,1986). However, Parthasarathy and coworkers (1986a) have presented compelling evidence which suggests that lipid laden foam cells are actually derived from tissue macrophages.

1.2.2 Blood-Borne Cells

1.2.2.1 The Platelet

Under normal physiological conditions, circulating blood platelets will not adhere to the vessel wall or each other. If however, they encounter a de-endothelialized region, platelets will be quickly activated leading to adhesion and aggregation. This process is important for hemostasis following injury through the formation of the platelet plug and has also been implicated in atherogenesis (for a review see Zucker, 1985). In vitro, platelets may be activated by a host of agents including ADP (Bennet and Vilaire, 1979), epinephrine, thrombin (DiMinno and Silver, 1983), prostaglandin endoperoxides and thromboxane A2, platelet activating factor as well as collagen (Marcus et al, 1981). The exact sequences of events in vivo still remains to be established. Activation causes platelet shape to change from smooth discs to sticky spheres displaying many filopodia. Stimulated platelets undergo degranulation releasing the contents from dense granules, primarily serotonin, and alpha-granule contents which include PDGF, factor V, fibrinogen, von Willebrand's factor and throbodopsin (Sixma and Webster, 1977). Aggregated platelets are finally enmeshed in a lattice-work through the conversion of fibrinogen to fibrin by thrombin.

Platelets are readily activated by a variety of agents. Further, these cells represent a source of biologically active substances. Several studies have implicated these blood-borne cells in atherogenesis. Normal platelet function is impaired in swine with von Willebrand's disease due to the absence of von Willebrand's factor

(Moake et al , 1980) and these animals are highly resistant to experimentally induced atherosclerosis.

Many platlet constituents have been shown to be chemotactic for cells in the immediate microenvironment of the artery wall. Platelet factor IV has been shown to be chemotactic for macrophages (Dueul et al, 1981) and PDGF has been found to be both chemotactic (Grotendorst et al, 1982) and mitogenic for SMCs (Ross et al, 1974, Ross, 1986) and all cells of mesenchymal origin (Huang et al, 1984). Fairbanks (1986) showed that PDGF promotes increased synthesis of HMG CoA reductase the regulatory enzyme of cholesterol synthesis, in fibroblasts. This suggests that this growth factor may have the potential to alter normal cholesterol metabolism in the artery wall.

1.2.2.2 The Macrophage

The monocytes are actively phagocytic. They enter the circulation upon release from the bone marrow and circulate for approximately 24 hours. After this point the monocyte enters the tissue where it is commonly referred to as a tissue macrophage. The Kupffer cell found in the liver and the alveolar macrophages of the lung are specialized forms of the tissue macrophage derived from the blood monocytes. Functions attributed to the tissue macropgage system are also described under the heading of the reticuloendothelial system (Natham et al,1980).

A growing body of evidence suggests that the macrophage cell is the putative precursor to the lipid-laden foam cell, a feature in both the fatty streak and mature grumous plaque (Fowler et al,1979).

Electron microscopic evaluation of lipid-laden cells from atherosclerotic lesions have been conducted. However these studies provided little insight into the identity of the cell type since such engorged cells do not retain many of their ultrastructural features (Parker and Odland, 1966). In vitro studies demonstrated the potential of intimal SMC to accumulate large amounts of lipid and thus take on a foam cell-like appearance. Lipid inclusions of macrophage origin (Wolfbauer et al, 1986) were used successfully to load SMC and induce the morphology of the foam cell. Fowler (1979) separated SMCs and so-called foam cells from atherosclerotic arteries and demonstrated the presence of the Fc and C3 receptors which are known to be present on monocytes. Other investigators using a variety of approaches have also presented evidence supporting the role of the monocyte as the progenitor to the foam cell (Schraffner et al, 1980).

While the events that give rise to foam cell formation in vivo are far from clear, a great deal of information regarding the behavior of these cells in vitro has been collected. In addition to their well known phagocytic activity, these cells possess and secrete an array of biologically active substances including free radical species and several potent products derived from the arachidonic acid pathway, neutral and acid hydrolases, coagulation and complement factors (Nathan et al, 1980) and macrophage derived growth factor, mitogenic for SMCs (Martin et al, 1981). Of considerable interest is the observation that macrophage cells possess a receptor for altered or modified LDL. Normal uptake of LDL does proceed by means of the apo B-E receptor described by Goldstein et al, (1979). The receptor which binds

modified forms of LDL has come to be known as the scavenger or acetyl-LDL receptor (Brown et al, 1980). This receptor is not down-regulated in the presence of excess ligand and thus represents an avenue for lipid accumulation and foam cell development. The potential importance of identifying this scavenger receptor becomes clear in light of the intriguing observations that LDL incubated in the presence of endothelial cells (Henriksen et al, 1981), SMCs (Heinecke et al, 1984) or exposed to the platelet product, malondialdehyde (Folgelman et al, 1980) is avidly taken up by macrophages. Even the macrophage cell itself can modify LDL through a free radical mediated process such that it is recognized by its own scavenger receptor (Parthasarathy et al, 1986, 1986a). This modification process has been shown to be due to lipid peroxidation with a requirement for metal ions of copper or iron which are known to mediate free radical reactions. The presence of antioxidants, or radical scavengers will curtail the modification of LDL (Parthasarathy et al, 1986).

1.3 Cholesterol and Atherogenesis

1.3.1 Introduction

The presence of massive lipid accumulation in the atherosclerotic artery has been well documented (Koletsky and Snajar, 1981). Lipid inclusions have been shown to consist primarily of cholesterol and its esters (Kruth, 1984) the majority of which is believed to originate from LDL (Bratzler et al, 1977) though an alternative process of cholesterol exchange may also be a contributing factor (Stender and Zilversmit, 1981). Considerable effort has been made to understand how total body cholesterol levels are controlled and in regard to atherogenesis, particular interest has focused on cholesterol fluxes within the microenvironment of the artery wall.

In addition to being the essential precursor for bile acids, corticosteroids, vitamin D derived hormones and the sex hormones, cholesterol along with PC make up the major structural components of cell membranes. The compartmentalization of discrete cellular functions as well as support matrix for membrane associated proteins are important fundamental roles served by membranes. The functional significance of cholesterol in membranes is not well understood. Physical studies in synthetic membrane model systems have provided evidence to suggest that cholesterol acts to decrease membrane fluidity above the lipid phase transition (Shinitsky, 1984). A recent study has demonstrated that changes in membrane fluidity in microsomes can modulate the activity of the ER bound enzyme HMG-CoA reductase (Davis and Poznansky, 1986).

From the simple two carbon precursor, acetyl-CoA, some thirty reactions culminate in the production of the cholesterol molecule. All the steps in this synthetic pathway have been elucidated. The production of mevalonate from hydroxymethylglutaryl-CoA is catylysed by the enzyme HMG-CoA reductase and represents the committed and rate-limiting step in cholesterol biosynthesis (Bloch, 1965).

It has been known for some time that cholesterol synthesis in liver and intestine are subject to stringent regulation and in vitro studies on peripheral cells would also suggest that most cells possess a mechanism for the regulation of cholesterol biosynthesis. An important consideration in vivo stems from the appreciation that peripheral tissue cannot catabolize cholesterol and in this instance overall cellular cholesterol levels result from balancing incoming cholesterol and de novo synthesis with cholesterol efflux. Because cholesterol is highly insoluble in water its movement is achieved by circulating lipoprotein particles. The particle core consists of CE and TG in varying relative amounts. The surface of the particle is sheathed in a phospholipid monolayer and embedded protein. The protein associated with these particles are referred to as apoproteins. By means of these apoproteins, the particle is recognized by specific cell surface receptors which will be discussed in detail below. Lipoproteins are separated by preparative ultracentrifugation and classified by their flotation densities. The major classes include HDL, which has been implicated in mediating cholesterol removal from the periphery, IDL and VLDL. The low density lipoprotein, LDL contains the greatest relative amount of cholesterol and functions to deliver cholesterol to peripheral tissues.

1.3.1.1 LDL-Receptor-Mediated Regulation of Cholesterol

Perhaps the most powerful evidence to date linking elevated blood cholesterol levels and risk of CVD has emerged from a series of elegant studies which elucidated the process of LDL uptake and degradation in normal cultured fibroblasts and its impairment in fibroblasts from individuals with Familial Hypercholesterolemia (FH). This disease was first described by Carl Muller in 1938 who noted that afflicted patients had elevated blood-cholesterol levels and myocardial infarction very early in life (Brown and Goldstein, 1986). Work by Goldstein and Brown (1973), showed that normal uptake of LDL is mediated by a specific cell surface receptor and in FH patients, the receptor activity is defective. This led to the development of the LDL Receptor Hypothesis for cellular free cholesterol regulation (Goldstein et al, 1979a).

An important preliminary finding in this study was the observation that cells grown in the absence of lipoproteins rapidly increase their HMG-CoA reductase activity, the rate limiting step in cholesterol synthesis. Through the addition of serum or LDL, this increase could be reversed. (Brown et al, 1973). It was proposed that cholesterol biosynthesis in nonhepatic cells is under constant feedback inhibition by cholesterol carried in LDL and acting at the level of HMG-CoA reductase. Complimentary work showed that this control mechanism acts by altering the rate of enzyme synthesis (Brown et al, 1973, Edwards and Gould, 1972).

The regulatory consequences of this model are summarized as follows:

The LDL particle binds to the specific cell surface receptor through recognition of the apoprotein component, apoB. Bound LDL-receptor complex is endocytosed and at the level of the lysosome, the LDL CE is hydrolysed to free cholesterol and fatty acid. The free cholesterol may then be used for the synthesis of membranes or re-esterified for storage. This process elicits three regulatory events within the cell.

The first is suppression of HMG-CoA reductase, the rate limiting step in cholesterol synthesis and thus reduced de novo cholesterol production. The enzyme responsible for cholesterol esterification acyl CoA: cholesterol: acyltransferase is stimulated and finally LDL receptor synthesis is down-regulated giving rise to fewer receptors emerging at the cell surface.

For cells cultured in the presence of serum therefore, cholesterol needed for membrane synthesis is provided in the form of LDL. Rates of de novo synthesis remain low with excess free cholesterol esterified. LDL receptors are presented to the external medium in numbers appopriate to ensure a sufficient and not excessive amount of incoming LDL cholesterol.

Other investigators have since presented evidence that the enzyme may be subject to modulation by a process of phosphorylation/dephosphorylation (Scallen and Sanghvi,1983) or by fluidity within the ER membrane (Vanketsen and Mitropolous, 1982, Davis and Poznansky, 1987).

Studies continued using normal and FH fibroblasts to determine the molecular basis through which LDL exerts its regulatory action on cellular cholesterol biosynthesis. The inability of individuals with FH to take up and process LDL gives rise to cholesterol accumulation within the circulation that may reach in excess of 625 mg/dl in homozygotes compared to the normal level of approximately 200 mg/dl. The suggestion of defective LDL delivery was confirmed with the demonstration of impaired binding and degradation of ^{125}I-LDL in FH fibroblasts (Brown and Goldstein, 1974). Binding in normal fibroblasts could be inhibited with pronase and this coupled with the fact that only the high affinity, saturable component of total ^{125}I-LDL binding was defective set the stage for Brown and Goldstein (1974) to propose the existence of a cell surface receptor specific for LDL and the receptor mediated endocytic pathway model for regulation of cellular free cholesterol (Goldstein and Brown, 1979).

1.4 Phospholipids

1.4.1 Introduction

1.4.1.1 Phospholipids in Membranes

Phospholipids along with cholesterol are the primary constituents of membranes. The two opposing lipid leaflets make up the membrane bilayer which serves as a permeability barrier imparting cellular compartmentalization and support matrix for lipid bound proteins. Biological membranes are fluid in nature which permits lateral diffusion of lipid and protein in the plane of the membrane (Singer and Nicholson, 1972).

An important characteristic of the phospholipids is their amphipathic nature. They posses a polar hydrophylic head group and nonpolar hydrophobic fatty acyl tails. Arranged in a bilayer, phospholipids orient in such a way that their polar regions face outward contacting the aqueous phase while the hydrophobic regions aggregate excluding water.

In eukaryotic membranes, the common glycerol-based phospholipids include PC, PE, PS and PI along with cardiolipin. Sphinogsine based sphingolipids such as sphingomyelin are also present.

Lipid structure and distribution is complex. The general name phosphatidylcholine includes a variety of PC species that are specifically identified by the fatty acyl chains residing at the sn-1 and sn-2 positions of the glycerol backbone. For the majority of phospholipids in mammalian membranes, saturated fatty acids are esterified at the 1-carbon while unsaturated species occupy the 2-carbon position (Vance, 1985).

Lipid composition also varies depending on the cell organelle and are commonly distributed asymmetrically in the two lipid leaflets comprising the same membrane (Op den Kamp, 1979). In the plasma membrane of red blood cells the majority of PS and PE are found in the inner or cytosolic monolayer while the reverse is true for PC and SM which occur in greater relative amounts in the outer monolayer. The functional significance as well as the mechanism responsible for establishing and maintaining membrane asymmetry is unknown.

1.4.1.2 Phospholipid Synthesis and Regulation

While many aspects concerning the synthesis and regulation of cholesterol have been studied in great detail, much less is known about factors governing the production of phospholipids. It is known however, that cells do attempt to maintain a fixed cholesterol to phospholipid ratio (Sandermann, 1978). It has also been demonstrated that LDL will down-regulate endogenous PC synthesis in fibroblast cells (Poznansky et al, unpublished) though the underlying mechanism mediating these observations remains to be elucidated.

The most abundant phospholipid species in membranes is PC. The major route of de novo PC synthesis proceeds by way of the CDP-choline pathway utilizing glycerophosphate, fatty acids and choline derived from the diet. In the mid 50s it was shown that PC synthesis required CTP mediating the enzymatic activity of CTP:phosphocholine cytidylyltransferase and CDP-choline:1,2-diacylglycerol phosphocholine (Kennedy, 1956). De novo synthesis of the other phospholipids also requires CTP and will be discussed below. A second pathway, of quantitative importance only in liver gives rise to PC from PE. This is

referred to as the methylation pathway using S-adenosylmethionine as the methyl group donor. Modification in the fatty acyl chains of PC has been described by Lands (1960). This involves a process of deacylation and reacylation occuring primarily at the sn-2 position of the PC molecule. The Lands pathway may contribute to PC turnover however its relative importance in vivo is largely unknown.

The enzyme responsible for the regulation of PC synthesis has been shown to be CTP:phosphocholine cytidylyltransferase. It has been found both in the cytosol and in microsomes in rat liver and requires lipid for activity (Pelech and Vance, 1984). In recent studies using HeLa cells it has been demonstrated that the primary site of PC synthesis is on the ER membrane while the cytosolic cytidylyltransferase exists as an inactive form (Vance et al, 1980). Translocation to the ER activates the enzyme and provides a means for rapid PC synthesis without the need for synthesis of new enzyme. Regulation of enzyme translocation and thus PC synthesis is mediated by at least two processes. The first involves covalent modification through a mechanism of phosphorylation/dephosphorylation shown to occur in the liver of rat. Inactivation is achieved by cAMP-dependent protein kinase. Fatty acylCoAs will also induce synthesis, not by acting on the enzyme directly but by inducing the translocation to the ER membrane (Vance and Pelech, 1984).

The synthesis of the other phospholipids is less well understood. De novo synthesis of PE involves the enzyme CTP:phosphoethanolamine cytidylyltransferase. In liver it occurs in the cytosol though there has been no evidence of activity in microsomes. Further, it doesn't

show any requirement for lipid as is the case for the PC equivalent. Nevertheless there is evidence to suggest that this enzyme is the rate limiting step in PE synthesis (Sundler and Akesson, 1975).

1.4.2 Biologically Active Lipids

Membrane lipids have long been thought to play a passive role providing cellular partitioning, with the normal cellular activities being mediated primarily by proteins (Bretscher, 1973). A renewed interest in the role of lipids has emerged with the demonstration of the potent biologically active metabolites of AA, found primarily in phospholipids (Moncada, 1977). The demonstration that platelets may be activated by an unusual lipid termed platelet activating factor (Benveniste et al, 1972) and a role for PI in signal transduction across the plasma membrane mediating a host of intercellular events (Berridge, 1984), represent active processes involving phospholipids.

1.4.2.1 The Eicosenoids and the Arachidonic Acid Cascade

The 20 carbon acids are refered to as the eicosenoids with AA (5-cis-,8-cis-,11-cis-,14-cis-eicosatetraenoic acid) being the most abundant representative. The other eicosenoids include 8-cis-,11-cis-,14-cis-eicosatrienoic acid and 5-cis-,8-cis-,11-cis-,14-cis-,17-cis-eicosapentaenoic acid or EPA which has been the focus of considerable interest and will be discussed further.

The arachidonic acid cascade provides two potential routes for generating a host of biologically potent substances. The cyclooygenase

pathway gives rise to the prostaglandins and thromboxanes while lipoxygenase pathway generates hydroxy or hydroperoxy eicosanoic acids and the leukotrienes (Smith and Borgeat, 1985).

Products of the cyclo-oxygenase pathway are referred to as autocoids or local hormones since their action occurs near sites of synthesis. EC, SMC and platelets all generate prostanoids. Anti-inflammatory agents such as aspirin inhibit prostaglandin synthesis by competing with AA for the active site on the cyclo-oxygenase enzyme. While prostanoid synthesis and chemistry is reasonably well understood, less is known about the regulation and physiological function of these compounds (Stehle, 1982). Cyclo-oxygenase products are not stored as is the case for many hormones, but are synthesized in bursts in response to certain stimuli, tapping precursor AA from membrane phospholipids. AA release may be elicited in response to angiotensin II, epinephrine and thrombin through specific receptors on a given cell type. In vitro, Ca^{+2} ionophores such as A23187, membrane active venoms or phorbol esters will elicite AA release as well as other fatty acids residing at the sn-2 position of phospholipids.

In the artery both prostacyclin and prostaglandin E2 are produced by SMC and endothelial cells and mediate the process of vasodilation (Moncada et al,1976). The major cyclo-oxygenase product in platelets, thromboxane A2, is the most potent platelet activator yet described. Prostacyclin is the primary prostanoid produced by endothelial cells and functions to inhibit platelet activation and aggregation. It has been suggested that balanced production of these compounds between these two cell types may play an important role in limiting

platelet-endothelial cell interactions within the artery (Moncada and Vane, 1980).

Recently, eicosapentanoic acid or EPA, another of the c-20 fatty acids has received considerable attention with the observation that Greenland Eskimos with an EPA rich diet show low incidence of CVD (Bang et al, 1980) increased levels of EPA in serum and platelets (Dyerberg et al, 1978) and reduced platelet aggregation. Evidence suggests that EPA reduces AA levels in platelets which in turn reduces thromboxane A2 production (Nagakawa, et al, 1983). In other studies evidence was presented that suggests that PGI2 production in EC and SMC may be reduced by linoleic acid thus favoring platelet aggregation and the mechanism is believed to be due to competition with AA (Morisaki et al,1985).

1.4.2.2 Phosphatidylinositol Cycle

The rapid turnover of phosphatidylinositol (PI) was first described by Hokin and Hokin (1953). Little developed from this early obsevation until more recently. A renewed interest in PI turnover grew from the observation of PI mediated signal transduction across the plasma membrane. The protein kinase C system has been described in a variety of cell types and represents a Ca^{++} requiring second messenger, the function of which is dependant on membrane lipids. Protein kinase C in turn functions to phosphorylate proteins and thus influences cellular processes (Nishizuka et al, 1984). A large body of evidence suggests that protein kinase C function is mediated directly by the turnover of PI in membranes. In response to an extracellular stimulus, this process liberates IP3 and DG which enhances protein

kinase C sensitivity to Ca^{++} giving rise to increased kinase activity without the need for increased intracellular Ca^{++}. The tumor promoting phorbol esters perform a function similar to DG and also activate protein kinase C (Costagna et al, 1982).

1.4.3 Effect of Membrane Fluidity

An important parameter used to study membrane phospholipid behavior is the concept of fluidity. At physiological temperatures, membrane phospholipids exist exclusively in a fluid or liquid-crystalline state. A reduction in temperature will cause phospholipids to undergo a phase transition to the more ordered gel state with a sharp decrease in mobility. This point is referred to as the phase transition temperature and is unique for a given phospholipid species. Phospholipids with lower phase transition temperatures will be more fluid at physiological temperature than those with higher transition temperatures, this variability due largely to the fatty acyl constituents in a particular phospholipid. Biological membranes contain many phospholipid species within a single class. For PC there will be several unique species depending on the acyl chain composition and location on the PC molecule. The fluid nature of mixed phospholipids in a biological membrane is a reflection of the total lipid acyl chain composition.

In general, long saturated acyl species will pack more readily and thus be less fluid than shorter or unsaturated fatty acyl chains. Alteration in membrane fluidity by modifying acyl chain composition has been used successfully to study synthetic model membranes as well as functional changes in biological membranes (Stubs and Smith, 1984).

By altering acyl chain composition in response to events such as temperature variation, bacteria adjust membrane fluidity and thus maintain membrane protein function. Cholesterol present in the animal cell represents an alternative means of regulating fluidity (Kimelberg and Papahadjopoulos, 1974). At 37 degrees cholesterol orders adjacent acyl chains in phospholipids, reducing mobility and thus fluidity. DPPC liposomes are more permeable to glucose if cholesterol is included in the bilayer (Inoue, 1974). It has been suggested that the loss of contact inhibition in virally transformed cells may be related to the observation that such cells have a lower cholesterol/phospholipid ratio (C/P) resulting in membranes with a more fluid nature (Barnett et al, 1974). A reduction in the C/P ratio in malignant lymphoma cells from mice and humans has also been reported (Inbar, 1974).

The concept of fluidity in membranes may be a potentially important consideration in our understanding of normal cellular function. Acyl chain modification coupled with the rigidifying action of cholesterol each contibute in dictating membrane fluidity. To an extent, increases in cholesterol will be offset by greater incorporation of unsaturated acyl species into membrane phospholipids. However, if this compensatory mechanism is overloaded, excess cholesterol may be accomodated simply by increasing total membrane which may be an underlying contributor to cellular proliferation in general and that seen in the artery in particular (Jackson and Gotto, 1976).

1.5 Phospholipases

1.5.1 Introduction

The PI second messenger system, modulation of membrane fluidity and the AA cascade are mediated by fatty acyl moieties of phospholipid molecules. A common theme in the study of these systems has been the recognition that fatty acid modification or release represents an important point of regulation mediated largely through the activity of a class of enzymes referred to as phospholipases.

1.5.2 Phospholipase Species

The demonstration of enzymes that hydrolyse phospholipids was described many years ago with the observation that fatty acids were released by PC incubated with pancreatic juice. These naturally occuring enzymes were termed phospholipases and are unusual in that they function at the water-lipid interface. Phospholipase activity has been described in bacteria, insects and many mammaliam tissues and cells including platelets and macrophages (van den Bosch, 1980).

Phospholipase A hydrolyses fatty acyl chains from phospholipids. Phospholipase A1 acts at the sn-1 acyl ester bond on the glycerol backbone while phospholipase A2 functions only at the sn-2 location. Other phospholipases have been described which act preferentially on lysophospholipids (hydrolysis of the single acyl chain) while another designated phospholipase B will attack both the sn-1 and sn-2 ester bonds. On the sn-3 carbon, the

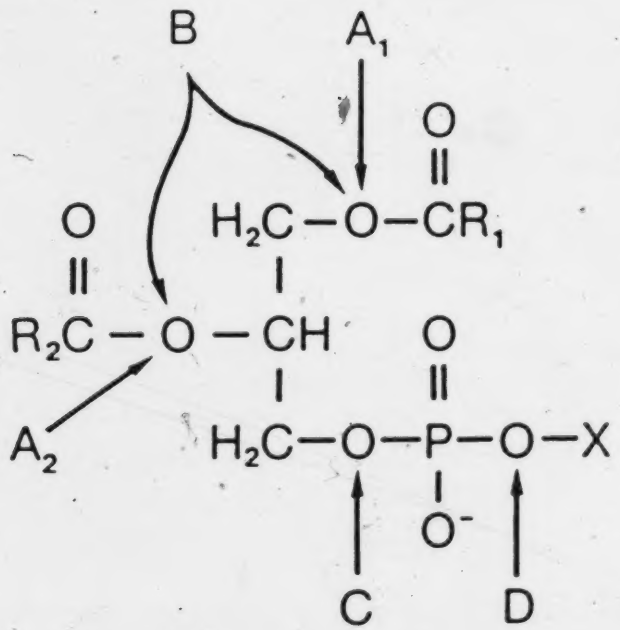
glycerol-phosphate bond is the specific site of attack of phospholipase C while phospholipase D mediates head group removal leaving the phosphatidic acid form of the phospholipid. The preferred site of phosphlipase activity is presented in Figure 2.

Certain of these phospholipases are thought to play a role in the lipid mediated processes described earlier. Phospholipase A 2 activity is required for the release of AA and eicosenoid production while phospholipase C acting on PI produces DG and admits insositol into the PI cycle. The mechanism behind deacylation-reacylation also requires phospholipase activity, thought to be primarily the function of the A2 form.

While it is clear that positionally specific release of fatty acids participates in several cellular processes mediated by phospholipases, the regulatory mechanism controlling such release is not well understood. Evidence has been presented which shows that phospholipase activity may be blocked by proteins produced in cells stimulated by steroids. These proteins have been given the name lipomodulin (Daniel, 1985). For the most part however, the physiological function of the phospholipases remains to be elucidated.

For the study of phospholipids, the array of positionally specific phospholipases are a valuable analytical tool. A great deal remains to be addressed concerning fatty acid and phospholipid synthesis and regulation. Coupled with radio-labled precursors and an ability to enzymatically dissect the phospholipid molecule may provide new insight into phospholipid metabolism.

FIGURE 2. Cleavage Sites of the Phospholipases.



1.6 Fatty Acid Turnover

Deacylation and reacylation or fatty acid turnover is a process that mediates acyl chain compositional changes in phospholipids, AA release and the PI cycle. As stated above the turnover process implies the activity of phospholipases. While it is generally accepted that de novo phospholipid synthesis occurs at the level of the ER there is some evidence to suggest that phospholipid modification is achieved at least in part at the level of the plasma membrane (PM). As a mode for communicating extracellular signals to the cell interior, the PI cycle by design must be occurring at the PM. However the PM may well be equiped to synthesize and modify phospholipids making the notion that phospholipid synthesis exclusive to the ER untenable (Chakravarthy et al,1986).

Many gaps exist in our knowledge of phospholipid synthesis, compositional changes of acyl chain constituents and the precise cellular location in which these processes take place. The potential importance of these processes is only beginning to be appreciated while mechanisms to explain their regulation remain unknown.

1.7 Theories of Atherogenesis

There are two schools of thought concerning the development of atherosclerosis. The most long standing approach is described under the heading of the Lipid Hypothesis. It has been known for many years that lipid-rich diets will induce plaque development in experimental animals as well as in man. This theory received considerable impetus with the elucidation of the receptor mediated endocytosis of LDL in cholesterol homeostasis described by Brown and Goldstein (1974) and the observation of premature atherosclerosis in individuals with impaired receptor function as seen in FH. Further genetic evidence in the form of the Watanabe rabbit, an animal model of the receptor deficiency state also showing development of early atherosclerosis underscores the role of altered lipoprotein processing and atherogenesis (Watanabe, 1980).

However, the question of why plaques develop where they do is not readily explained within the framework of the Lipid Hypothesis. As a consequence, Ross and Glomsett (1976) proposed the 'Response to Injury' hypothesis which states that injury to the endothelium at specific sites in the arterial tree may set the process of atherosclerosis in motion. With minor modification, both approaches have been strengthened with continued investigation and recently it has been suggested that the two hypothesis may not be mutually exclusive (Steinberg, 1983).

A unified hypothesis proposed by Steinberg (1983) has incorporated recent observations concerning growth factors produced by EC, SMC and platelets and aspect of lipoprotein processing by

macrophages. This has led to the suggestion that the lipid-response to injury hypotheses may be interelated at many points. There is evidence to suggest that elevated LDL levels may damage endothelium (Ross and Harker, 1976) as well as increase platelet aggregation (Shattil, 1975). That endothelial loss favors greater infiltration and degradation of lipoproteins has been demonstrated in several studies (Ramirez et al, 1984, Alavi and Moore, 1984, Peng et al, 1985). Increased platelet reactivity may favor the release of PDGF which has been shown to stimulate SMCs and increase the expression of LDL receptors on these cells (Chait et al,1980). Finally, stimulated SMCs produce excess extracellular matrix material such as glycosaminoglycans (Alavi and Moore, 1987), which may favor retention of LDL within the artery wall (Ross and Klebanoff, 1971). Steinberg points out that an appreciation of these relationships may be needed to address the many questions that remain in understanding atherogenesis.

1.8 Endothelial removal and compositional changes to the artery

1.8.1 Problems and experimental approaches

The endothelium was once thought to be a passive barrier. It is now accepted that the endothelial cells play an active role in mediating the flux of materials from the plasma compartment contributing to the environment within the artery wall. The 'response to injury' theory of atherogenesis suggests that injury or loss of endothelium precipitates the development of intimal lesions (Ross and Glomsett, 1976). Exposure of the subendothelium promotes platelet aggregation and release of mitogens into the underlying cells of the artery wall. Consequently there has been considerable interest in characterizing the EC and identifying factors that may be injurious to endothelium in vivo. Effort has also been made in describing functional and compositional changes to the artery in response to experimental de-endothelialization.

In studies on de-endothelialized arteries, an important issue is the demonstration that the method used for endothelial removal in no way damages the underlying elastic lamina or medial layer of the artery wall. Investigators have used freezing, heating, clamping and chemical irritants to induce selective injury to the artery wall. However, in none of these procedures was it possoble to control the depth of injury (French,1966). Bjokerud (1969) used a looped wire with an abrasive surface to scrap the endothelium from the lumen. However, this technique was shown to tear the internal elastic lamina and degradation to SMCs was also demonstrated. This is an important consideration in light of recent evidence which has shown that

repeated injury to the intima will cause lesions composed of platelet-fibrin thrombi. These lesions will regress if the injury stimulus is removed. Alternatively, progressive development of lipid rich lesions are favoured by a single injury event restricted to the endothelium (Moore et al, 1982).

The use of balloon catheterization has been adopted by many investigators. This technique removes only the endothelium and minimizes the possibility of damage to the underlying tissues of the artery (Spaet et al, 1978). Gorog (1985) has also selectively removed endothelium in rat carotid artery by laser irradiation.

That endothelial loss alters artery wall function has been described in several studies. Changes in artery wall permeability due to de-endothelialization have been observed in arterial segments of rabbit perfused in situ. An immediate increase in the flux of labelled glucose and albumin was observed in de-endothelialized arteries relative to controls and slight increase in the eflux of tritiated water was also observed. Greater permeability was detected in segments from animals that were mintained on a high cholesterol diet (Chobanian et al, 1983). Enhanced uptake of radiolabelled LDL by the de-endothelialized artery has also been described (Bratzler, 1977). In long term studies, de-endothelialized regions initially showed an increase in permeability to LDL which dropped to near normal levels after 24 hours. In areas of endothelial regrowth, enhanced and persistent accumulation LDL was observed (Alavi and Moore, 1984). Further work provided evidence to suggest that permeability to LDL is increased in regions of endothelial regrowth (Day et al, 1985).

A host of compositional alterations have also been described in the de-endothelialized artery. Placement of indwelling catheters in rabbit aortas for a 2 week period induced an increase in the content of cholesterol and cholesterol ester. Incorporation of ^{32}P into phospholipid demonstrated greater uptake into PC with a reduction in PI when compared to regions of artery with intact endothelium (Day et al, 1974). Lipid evaluation was also conducted in chronic studies in de-endothelialized rabbit aorta examining compositional changes in denuded areas and in regions of endothelial regeneration. A generalized increase in lipid content was detected in areas of endothelial regrowth. Cholesterol content of denuded areas was maximal at 6 months and persisted to 24 months. In those regions covered with regenerated endothelial cells, greater cholesterol content was also apparent at 6 months and continued to increase up to 24 months. CE content was also shown to be greatest in areas of endothelial regeneration. Total lipid phosphorous indicated increased phospholipid content beneath regenerated endothelium while phospholipid content of denuded areas parallelled that of uninjured areas.

There is increasing evidence to suggest that GAGs may also play an important role in lipid accumulation in the artery wall. In isolated human atherosclerotic aorta it was demonstrated that only 30-40% of trapped lipoprotein could be recovered from lesions using conventional extraction procedures. Digestion of the tissue with collagenase, elastase and chondroitinase facilitated the release of the residual lipoportein. This observation suggests that acid mucopolysacharides may bind lipoproteins within the artery wall

(Hollander, 1976). The potential of GAGs to form complexes with LDL in vitro has been described by Iverius (1972). Injury to the aorta in rabbit also results in alterations in GAG content within the artery wall. At 11 and 17 weeks after de-endothelialization, denuded areas showed GAG and lipid content similar to uninjured regions of the artery. A marked increase in GAG and lipid content was apparent within the artery wall covered with a regenerated endothelium (Richardson et al, 1980). An important question pertaining to atherogenesis is whether the increase in GAG content occurs before lipid accumulation. Recently evidence has been presented in support of such a process. Aortic lesions were induced in aorta of rabbits by placement of indwelling catheters. Catheters were then removed and the process of regression was examined. It was observed that after 4 weeks proteoglycan concentration was maximal while lipid was still continuing to accumulate. As the lesions began to regress, the protoglycan content declined before any reduction in lipid content was apparent. These findings suggest that temporally, increased proteoglycan content may occur before changes in lipid. Thus greater proteoglycan content may promote retention of LDL within the artery wall resulting in lipid accumulation (Moore and Richardson, 1985). Additional investigation has demonstrated alterations in the composition of proteoglycans. In areas of endothelial regrowth, in addition to a net increase in proteoglycan content a marked increase in chondroitin sulphate and dermatan sulphate was detected while heparin sulphate content appeared similar to that found in areas of undamaged artery. It was also demonstrated that in animals fed a cholesterol rich diet, the increase in proteoglycan content and compositional changes were accentuated (Alavi and Moore, 1987).

1.9 Objectives

The purpose for conducting this study was twofold. The primary objective was to examine the short term alterations in lipid metabolism in rabbit carotid arteries with and without endothelium. Lipid compositional changes have been described in a host of studies on de-endothelialized arteries *in vivo*. For the phospholipids, several synthetic or reutiliztion pathways may be involved. The same may be said for cholesterol which may arise through *de novo* synthesis or by hydrolysis of stored CE. In arteries where a change in lipid content has occurred, it may be difficult to pinpoint the pathway responsible for such a change. In this study, the experimental interval was adequate to demonstrate a shift in lipid metabolism in response to de-endothelialization. The experiments were also sufficiently brief to permit the identification of the mechanism responsible for the observed change.

To carry out this study, a procedure was developed to remove and perfuse segments of rabbit carotid artery under pressure while maintaining an intact endothelium. The presence of an intact endothelial lining was demonstrated by perfusion-fixation and SEM. Fixation was carried out in the perfusion apparatus used for the metabolic studies. An additional procedure was developed to remove only the endothelium while introducing no detectable damage to the underlying artery wall viewed under SEM. Lipid metabolism was monitored by following the incorporation of radiolabelled precursors under a variety of experimental conditions.

2. MATERIALS AND METHODS

2.1 Materials

^{3}H-acetate, sodium salt (100mCi/mmol), ^{14}C-acetate, sodium salt (60 mCi/mmol), ^{3}H-choline (90mCi/mmol) and ^{35}S-methionine (1127 Ci/mmol) were obtained from New England Nuclear (Lachine, Quebec). Aqueous counting scintillant was purchased from Amersham Canada (Oakville, Ontario). Chloroform, methanol, hexane and heptane were purchased from Fisher Scientific Co. and were redistilled prior to use. Fetal calf serum was purchased from Flow Laboratories (Toronto, Ontario). Tissue perfusate media was obtained from Gibco Canada (Burlington, Ontario. Other chemicals were purchased from Sigma (St. Louis, Mo.). Silica Gel G [Sil G] plastic backed thin layer chromatograghy plates were purchased from Brinkman Canada (Rexdale, Ontario). Gas Liquid chromatography supplies and standards were obtained from Supelco (Oakville, Ontario) and electrophoresis materials were purchased from Biorad Laboratories (Richmond,Cal.). Rabbits used in this work were lop-eared Flemish giants of either sex from the Medical Science Building Animal Unit, University of Alberta, Edmonton, Alberta.

2.2 Perfusion Apparatus

The perfusion apparatus was designed to accomodate a maximum of eight arterial segments although six segments were generally used in the experiments described here. A schematic representation of the apparatus is presented in Figure 3. The reservoirs were designed with the outlet fitting positioned at the bottom of the reservoir housing to ensure a bubble-free flow of perfusate. The inlet from the return feed line was positioned to deliver perfusate back to the reservoir at a point slightly higher than the perfusate surface to allow perfusate to flow down into the reservoir. This arrangement promoted self-bleeding of any bubbles trapped during the surgical removal of the segments. This also permitted greater exposure of the perfusate to the 5% CO_2, 95% O_2 atmosphere maintaining pH at approximately 7.4. Details of the reservoir are presented in Figure 4. The reservoir bank was attached to only one length of the containment unit and water from an external warming bath circulated directly beneath ensuring that the perfusate in the reservoirs was maintained at a temperature of 37 ^{o}C. The outlet for the water bath was also specifically placed to minimize the chance of heating water mixing with perfusate in the event of an accidental surge from the water bath.

The organ bath was positioned directly below the reservoir bank such that individual reservoirs were positioned over a single organ bath. Again, a total of eight such vessels were built into the organ bath unit and six used in a typical experiment. The volume of each organ bath was approximately 190 ml. In the barrier experiments described below in which ^{3}H-acetate was added to the organ bath as

Figure 3. Schematic diagram of perfusion apparatus.

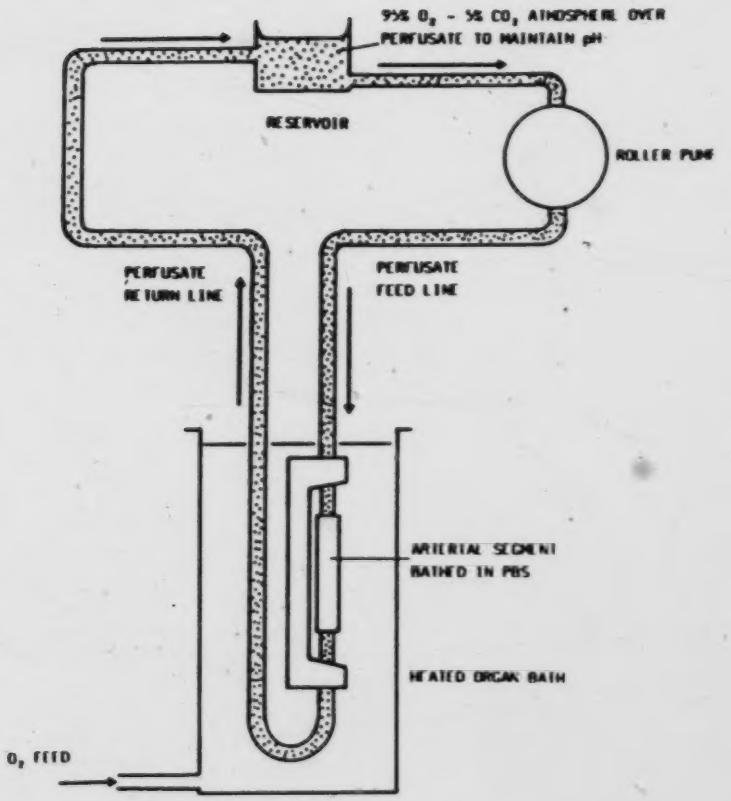
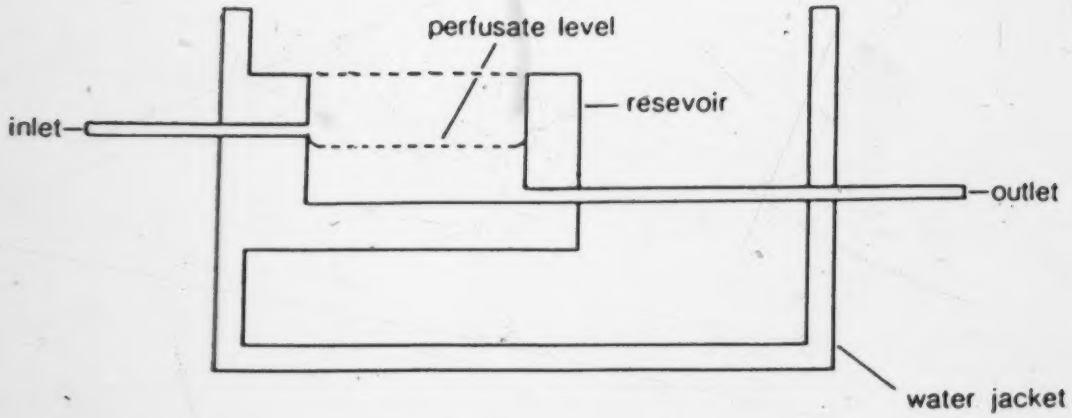


Figure 4. Detail of perfusion reservoir. High placement of return line purges bubbles from system. Low outlet ensures continuous bubble-free flow of perfusate.



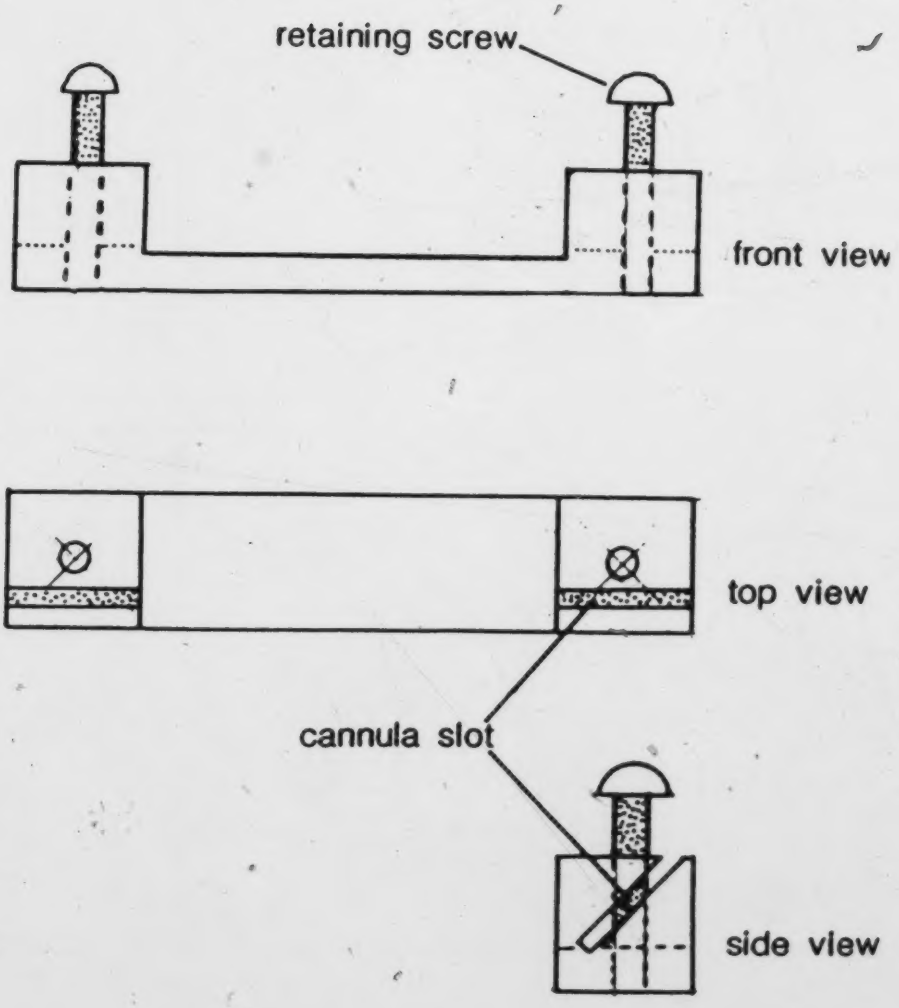
opposed to the perfusate, smaller organ baths were used to increase the concentration of ^{3}H-acetate presented to the outer surface of the arterial segments. These smaller baths held approximately 40 ml of PBS and reduced the amount of ^{3}H-acetate needed to conduct these experiments. All organ baths were supplied with oxygen through an inlet at the base. The water bath used to warm the reservoir bank was also used to control temperature of the organ bath unit with inlet and outlet again arranged to prevent overflow.

Perfusate was pumped at a rate of 1 ml/min by means of two four circuit roller pumps (Gilson Minipulse 2 using .032 in. internal diameter tubing) at a pressure of approximately 70 mm Hg.

Tissue Holders

In addition to preserving metabolically active arterial tissue and a healthy endothelial lining effort was made to maintain in vivo dimensions. Unrestrained segments of artery are highly elastic and will retract if permitted. This process will ultimately cause endothelial denudation due to the fact that retraction causes compression of the endothelial substrate and disruption of attachment points. Upon SEM evaluation such retracted segments showed substantial endothelial loss. To avoid this problem tissue holding devices were built and are represented schematically in Fig 5. Use of these holders is described below.

Figure 5. Detail of tissue holder.



2.3 Surgical Removal Of Arterial Segments

Lop-eared Flemish rabbits of either sex were used in this study. Animals were maintained on standard chow diet and weighed from 3 to 4 kg. These rabbits possess relatively large carotid arteries compared to other rabbit breeds. Anesthesia was produced by intravenous administration of approximately 0.25 ml sodium pentobarbital/kg. body weight. A ventral incision was made at the level of the jaw and continued posteriorly to the rib cage. Bleeding was minimized by cauterization. The underlying musculature was dissected aside to expose the carotid arteries. Of particular concern at this point was the identification of three to seven lateral branches leading away from the carotid. In order to perfuse segments these arterioles were tied off in early experiments to prevent leakage. Later cauterization was also used successfully in sealing these small branches. With the carotid lateral branches sealed over a length of approximately 10 cm any remaining membranous attachment was cut away. The animal was then placed on a small movable table and positioned over the perfusion apparatus which had been primed and readied. Segment removal was initiated by putting twelve loose ties along the length of the artery. These were used for securing the canula tips to the artery segments or simply as points of mechanical manipulation. Starting with the distal third of the artery, a feed line cannula was inserted, tied securely and an initial measurement made of the segment to be removed.

Proximal to this, a tie was secured leaving an intervening length of approximately 1-1.5 cm. The arterial segment ahead of the feed line was at this point perfused soley by means of the perfusion apparatus. Two cm downstream, the return line was inserted and the remaining carotid leading into the head region clamped. A tissue holder was then brought into position and the opposing cannula with the suspended intervening arterial segment secured in the holder. The isolated carotid segment was then cut free immediately proximal to the feed line insertion point and distal of the return line. Segment length was then remeasured and readjusted as required to its in vivo length and placed into the organ bath. The second and third segments were removed in a similar fashion with the exception that the return line was tied in first using the interval distal to the last tie as a starting point. With the insertion of the feed line, the intervening clamping tie was loosened or cut away to permit flow of feed line perfusate. The segment was cut free, mounted in a holder and measured as described above. The identical procedure was used on the remaining carotid giving rise to six individual perfused segments in total. In those segments designated as de-endothelialized, the supporting cannulae were advanced toward one another and then retracted. De-endothelialization was verified by scanning electron microscopy described below. The animal was euthanized by cardiac puncture. A mounted segment under perfusion is presented in Plate 1.

One difficulty with this procedure is that a bolus of native rabbit blood will occupy the second and third segments until artificial perfusion is established. This represents a potential source of

Plate 1. Placement of isolated arterial segment into tissue holder.

contamination particularily in the serum-free experiments. Therefore, perfusate was changed five times before endothelial removal. With the segments placed in the organ baths, perfusate reservoir levels were monitored to identify leaking segments.

2.4 PERFUSATE PREPARATION

In all the perfusion experiments described here, perfusate was made up of MEM alone, MEM plus 10% FCS or MEM plus 10% LPDS. In the serum dosage experiments, the percentage of LPDS varied from 2% to 10%.

Human LPDS (density >1.215 g/ml) and human LDL (density 1.091-1.063 g/ml) were prepared by differential ultracentrifugation of plasma from healthy volunteers (Hatch and Lees, 1968). Approximately 450 ml of venous blood was collected in a vessel containing disodium EDTA as an anticoagulant. Plasma was separated by centrifugation at 4,000 x g for 30 min at 4°C. and quickly used for lipoprotein isolation. Final plasma density was adjusted to 1.019 g/ml through the addition of potassium bromide and then centrifuged in a 70 Ti rotor using a Beckman ultracentrifuge at 50,000 rpm for 20 hours at 4 °C. The upper layer containing VLDL was discarded and the remaining sample was adjusted to a density of 1.063 g/ml with potassium bromide and centrifuged at 50,000 rpm for 28 hours at 4 °C. The LDL was collected from the top layer and the remainder of the sample adjusted to a density of 1.215 g/ml. After a final centrifugation at 55,000 for 48 hours, the LPDS fraction was removed by puncturing the bottom of the tubes and dialysed against 0.15 M-Na Cl/0.3 mM EDTA at pH 7.4.

2.5 Scanning Electron Microscopy

The objective of this study was the examination of early changes in artery wall metabolism in response to de-endothelialization. Therefore, it was of primary importance to demonstrate that an intact endothelial lining persisted in the control arterial segments. Any degree of depressurization or rough handling gave rise to partial or complete loss of the endothelial lining.

For the purpose of scanning electron microscopic evaluation, segments were perfusion-fixed using the same apparatus that was used for the metabolic studies. A fixative solution containing 2.5% glutaraldehyde in Millonigs' buffer (McDowell and Trump, 1976) was circulated for 60 minutes. The segments removed and split length-wise. The tissues were then dehydrated in graded ethanol for critical point drying, sputter coated with gold and viewed in a Philips Model 505 scanning electron microscope.

Segments designated with or without endothelium were examined by fixation immediately after installation into the perfusion apparatus, 20 or 60 minutes later or at 16 hours. Additional segments were allowed to depressurize in control segments to evaluate the effect of pressure loss on the endothelial lining.

2.6 Tissue Analysis

After the appropriate perfusion period, each arterial segment was removed from the perfusion apparatus and rinsed in normal saline. Individual segments were then placed into a tissue grinder and homogenized in one ml of methanol. Approximately 1,000 cpm ^{14}C-cholesterol was added as an internal standard and tissue lipid extracted using the method of Bligh and Dyer (1959). Lipid extract in chloroform was then evaporated under a stream of nitrogen and resuspended in a known volume of chloroform and aliquots used for the analyses described below. Neutral lipid separation was carried out using thin layer chromatography on Sil G TLC plates in a solvent system of hexane: ethyl ether: acetic acid (85:15:2). For polar lipid separation a solvent system of chloroform: methanol: acetic acid: water: (50:25:7:3) was used (Kates, 1982). Lipid spots were identified by co-migration with known lipid standards run on adjacent lanes of the TLC plates. Individual lipid spots were cut from the plates and added to 15 ml of scintillation cocktail for evaluation of radio-label incorporation. Polar lipid phosphorous content was determined for individual lipid species (Chalvardjian and Rudniki, 1970). Fatty acid content of PC , lysoPC and released fatty acid from the sn-2 position of PC (by PA2 hydrolysis, Haverkate and Van Deenen, 1965) was evaluated by transmethylation to the fatty acid methyl esters using 6% H_2SO_4 in methanol. Total tissue protein was determined as described by Lowry (1951) and ^{35}S-methionine incorporation into protein evaluated by scintillation counting.

2.6.1 Gas Liquid Chromatography

Fatty acid compositions of complete PC, lysoPC and the fatty acid released by hydrolysis with phospholipase A2 were determined. A Hewlett Packard 5730A gas chromatograph was used with 10% DEGS columns operated isothermally at 200 ^{o}C using hexane as the mobile phase. A known amount of C-17 fatty acid was included in the samples an an internal standard and peaks indentified by comparing retention times to those of prepared fatty acid methyl ester standards.

2.6.2 Phospholipase A2 Hydrolyss of Phosphatidylcholine

Fatty acid positional analysis of PC was conducted by hydrolysis with phospholipase A2. The procedure was initially standardized by hydrolysing prepared ^{3}H-DPPC labelled in both the sn-1 and sn-2 acyl chains and determining the radioactivity of the recovered products. Sample PC from control and de-endothelialized arteries was then subjected to the same procedure.

0.1 mg PC was added to 2 ml ethyl ether-methanol (98:2,v/v) in a 15 ml stoppered flask. Enzyme solution was prepared by dissolving 1 mg of lyophilized venom (Crotalus adamanteus) in a solution of 0.1M borate buffer (pH 7.0-7.4) containing 1.6 mg calcium acetate in a total volume of 5 ml. 200 microliters of enzyme solution was added to the dissolved PC and the reaction mixture shaken vigorously for thirty minutes at 25 ^{o}C. This process was repeated three times. At thirty minute intervals 50 microliters were removed and run on TLC plates. Generally, by 60 minutes, no residual PC could be identified on TLC plates when exposed to iodine vapor, the majority of stainable material

present was only in those spots corresponding to lysoPC and fatty acid. At 90 minutes, solvent evaporation was facilitated by addition of 0.5 ml benzene and the residue redissolved in chloroform-methanol (1:1) and products separated by TLC. For PC from arterial tissue the resuspended product solution was halved and run in two lanes of the TLC plate. One group was eluted and transmethylated for fatty acid analysis by GC while the other was prepared for scintillation counting for incorporation of radioactivity. Individual spots were identified by running known fatty acid, lysoPC and PC standards.

2.7 Experimental Design

2.7.1 Tissue Incubation

The first experiments were incubations of arterial segments in tissue culture medium. Approximately 2 cm sections of carotid artery were minced and placed in loosely stoppered test tubes containing 5 ml of serum-supplemented media. The tubes were mounted on a shaker table and placed into a tissue culture incubator. 100 uCi of ^{3}H-acetate was also added at the start of the experiments and the tissues incubated for 16 hours at 37 ^{o}C in 5% CO_2-95% O_2 atmosphere. Arterial tissue was then removed and evaluated for incorporation of radioactivity into lipid.

2.7.2 Cholesterol Biosynthesis in Perfused Arteries

The ability of arterial tissue to respond to removal of lipoproteins and thus up-regulate de novo cholesterol synthesis was determined by perfusing intact (with endothelium) segments with perfusate supplemented with FCS or LPDS. The time course for these experiments and the majority of the perfusion experiments described below was the following:

Segments were perfused for 12 hours at which time labelled precursors were added. The experiments were conducted for 16 hours at which time the segments were collected and analysed. Variations to this general protocol are noted where applicable.

2.7.3 Effect of Endothelial Removal in Perfused Arteries

This series of perfusion experiments examined the effect of de-endothelialization on lipid metabolism using a common perfusate preparation. In individual experiments, perfusate was supplemented with complete serum (FCS), LPDS or serum-free media. All of these experiments followed the standard time course described in section 2.7.2.

2.7.4 Movement of ^{3}H-acetate from Perfused Arteries

The appearance of radioactivity in the bathing buffer was monitored by sampling at the beginning of these experiments and at 4 hour intervals. 300 uCi of ^{3}H-acetate was added to the perfusate immediately after the segments were placed in the perfusion apparatus and the experiment conducted for 16 hours. Triplicate aliquots of bathing media were removed at each interval, each added to 15 ml scintillant and the extent of radioactivity reaching the bathing media was determined.

2.7.4.1 Appearance of ^{3}H-acetate in perfusate

Perfusate was unlabelled in these experiments. 300 uCi of ^{3}H-acetate added to the bathing medium. The organ bath vessels were miniaturized to 41 ml each to present ^{3}H-acetate to the adventitial side of the segments in greater concentration. Labelled acetate was included in the bathing buffer at the start of the perfusion and perfusate sampled at 4 hour intervals for 16 hours. Since the perfusate volume was relatively small, triplicate 25 microliter samples

were collected with the aid of a micro syringe. At each interval 75 microliters of fresh perfusate was returned to each reservoir to maintain a constant volume of 1 ml.

2.7.5 Effect of Increasing Serum Concentraction

Increasing serum concentrations (0-10% LPDS) were used to perfuse 6 de-endothelialized carotid segments. Perfusion was conducted for 16 hours with labelled precursors added at the 12 hours.

2.7.6 Short term perfusion

To examine the early effect of endothelial removal, experiments were conducted using abbreviated perfusion intervals. The periods examined were 0-2, 0-4, 0-8 and 0-12 hours. For the two longer perfusion experiments, labled precursors were added for the last 4 hours. Segments perfused for 2 hours only received ^{3}H-acetate at the onset of the experiment while the 0-4 series was pulsed for the last 2 hours only.

2.7.7 Labelling before endothelial removal

In one group of experiments, segments with endothelium were perfused with 10% LPDS in the presence of ^{3}H-acetate for an initial 8 hours. At this point three of the segments were de-endothelialized and perfusate in all 6 circuits replaced with fresh unlabled media . Thirty minutes later one control and one de-endothelialized segment were collected. Second and third pairs were removed in a similar fashion at 2 or 4 hours after de-endothelialization.

2.7.8 Double-labelling using ^{14}C-acetate and ^{3}H-choline

These experiments followed the standard protocol described in Sec. 2.7.2 with the addition of 25 uCi of ^{3}H-choline and ^{14}C-acetate. Precursors were added to perfusate after 12 hour of perfusion and segments collected at 16 hours.

2.7.9 Preparation of LPDS subfractions

The protein content of LPDS was successively reduced by adding increasing amounts of ammonium sulphate. LPDS-ammonium sulphate solutions were centrifuged for 15 minutes at 4000 x g at room temperature. Supernatant was collected and dialysed against three changes of 0.15M-NaCl/0.3mM-EDTA (pH 7.4) at 4 ^{o}C to remove residual salt. Dialysate was reduced by ultra-filtration to the initial starting volume and perfused through de-endothelialized arteries in the normal fashion.

2.7.10 In series perfusion of intact and de-endothelialized segments.

Four de-endothelialized and two intact carotid segments were placed in the perfusion apparatus. The return line of two control segments and the feed lines supplying two de-endothelialized segments were equiped with 't' fittings. Having verified that all the segments were leak free, a 10 cm length of perfusion line was inserted joining the t-fittings between one intact and one de-endothelialized segment. This resulted in perfusion in series of a control segment and down stream to it, a de-endothelialized segment. Two in series circuits were set up in this fashion. Segments were perfused for a total of 16 hours with labled precursors added for the last 4 hours.

2.8 Statistical analysis

Data were analyzed using the Student's 't' test (unpaired). Statistical significance was accepted if the P value was < 0.05. Results from individual experiments were presented in tabular form and represnt the mean of triplicate tissue samples. The pooled means for a group of experiments under a single condition are presented in the text. Variation from this format is noted where applicable.

3. RESULTS

3.1 Tissue Incubations

3.1.1 Cholesterol Biosynthesis in Minced Arterial Tissue

A primary concern was to determine whether endothelial loss had any effect on the synthesis of cholesterol. Therefore it was necessary to determine if arterial tissue retain the ability to regulate de novo cholesterol synthesis. Carotid segments were dissected, minced and incubated in tissue culture media containing either 10% FCS or 10% LPDS. It is known from in vitro studies that removal of lipoproteins specifically LDL promotes an increase in de novo cholesterol synthesis (Goldstein and Brown, 1973a). A preliminary series of experiments were done to determine whether minced arterial tissue remains capable of up-regulating cholesterol synthesis in the absence of LDL. The results from one experiment are summarized in Table 1. The mean cholesterol uptake of ^{3}H-acetate in 8 experiments were 1,946 $\pm$ 621 cpm/mg protein in FSC and 3,798 $\pm$ 1,083 in LPDS, $p < 0.05$. Segments incubated in LPDS for 16 hrs thus showed a modest but statistically significant increase in ^{3}H-acetate uptake into cholesterol when compared to similar arterial segments incubated in FCS. In tissue culture using cells such as fibroblasts, up regulation of cholesterol synthesis is also accompanied by a reduction in the rate of cholesterol esterification. No apparent reduction of cholesterol esterification was seen in the segments incubated in LPDS.

Table 1. Incorporation of ^{3}H-acetate into neutral and polar lipid in minced arterial tissue incubated for 16 hours in either 10% FCS or 10% LPDS in MEM. Lipids were extracted and counted as described in Methods. Results represent the mean ± S.D. of three samples from a single experiment and are representative of 8 experiments.
*Significantly different from values in tissue incubated with FCS ($p<0.05$)

	^{3}H-acetate incorporation (cpm/mg protein)	
	FCS	LPDS
neutral lipid		
polar origin	17,935 ± 3,930	19,645 ± 5,671
mono-diglycerides	5,128 ± 1,034	3,102 ± 1,005
cholesterol	2,366 ± 498	3,872 ± 257 *
free fatty acid	11,669 ± 2,647	12,767 ± 3,825
triglyceride	7,254 ± 1,549	4,899 ± 2,092
cholesterol ester	5,122 ± 1,715	4,899 ± 2,092
polar lipid		
PC	8,228 ± 1,113	7,696 ± 2,452
PE	6,434 ± 2,024	4,104 ± 2,105
SM	1,266 ± 681	2,789 ± 1,712
other	638 ± 290	815 ± 212

3.1.2 Incubation of Arterial Tissue With or Without Endothelium

A second series of experiments were performed in the same fashion as those described above with the exception that in half of the segments, the endothelium was removed. These de-endothelialized and intact segments were then minced and incubated in 10% FCS for the usual 16 hrs and analysed for ^{3}H-acetate incorporation into lipid. The results of one such experiment are summarized in Table 2, representative of 4 experiments conducted. No preferential uptake of label in de-endothelialized minced tissue was observed, in fact no shift in lipid metabolism could be seen in this series of experiments. While mincing the segments allows for gross comparisons of lipid metabolism using two different culture media types as described in the FCS-LPDS series above, comparison based on structural criteria was not possible. Immersion of whole or minced segments eliminates the sidedness of the tissue which is a feature of the tissue *in vivo*. Since there is no lumen there most surely is no endothelium proper in the control preparation. Secondly by evaluating perfused segments using scanning electron microscopy it was found that any degree of prolonged de-pressurization or rough handling of the arterial segments generally gave rise to partial or complete sloughing off of the endothelium. In this second series of minced arteries incubated with and without endothelium, it is likely that both the so-called intact preparations and the de-endothelialized tissue had no endothelium remaining.

Table 2. Incorporation of ^{3}H-acetate into neutral and polar lipid in minced tissue with or without endothelium. Tissue was incubated for 16 hours in 10% FCS in MEM. Lipids were extracted and counted as described in Methods. Results are the mean ± S.D. from one experiment and are typical of four experiments conducted.

	^{3}H-acetate incorporation (cpm/mg protein) with endothelium	without endothelium
neutral lipid		
polar origin	36,548 ± 4,305	33,436 ± 10,853
mono-diglyceride	9,341 ± 3,255	15,635 ± 7,052
cholesterol	6,843 ± 3,169	8,718 ± 2,582
free fatty acid	14,446 ± 6,324	13,086 ± 4,141
triglyceride	7,053 ± 3,527	8,998 ± 2,780
cholesterol ester	8,111 ± 3,605	7,998 ± 4,005
polar lipid		
PC	18,477 ± 2,137	16,226 ± 7,353
PE	7,384 ± 3,485	11,068 ± 4,238
SM	2,390 ± 1,587	2,668 ± 1,448
other	1,501 ± 1,299	2,302 ± 1,840

3.2 Experimental protocol

3.2.1 Time course

The study described here employed a novel method for isolation and perfusion of arterial segments under pressure. Although perfusion was carried out for as long as 24 hours, a general schedule of 16 hours was adopted. In the majority of the perfusion experiments, labelled precursors were added at the 12 hours and arterial segments collected for analysis at 16 hours. This time course was chosen for the following reasons. Perfusion of segments for the initial 12 hour period was necessary to identify those that were leaking. Those segments showing no drop in reservoir level at 12 hours were considered to be leak-free. At this point radioactive precursors were added. One objective was to determine whether perfused arterial segments retain the ability to regulate de novo cholesterol synthesis. This was examined by comparing ^{3}H-acetate incorporation into cholesterol in perfused segments using serum with lipoproteins (FCS) or lipoprotein deficient serum. From studies using cultured fibroblast cells, LPDS promoted an increase in de novo cholesterol synthesis relative to similar cells grown with FCS (Brown and Goldstein, 1973a). This increase is usually apparent 6 hours after lipoproteins are removed from the medium and reaches a maximum at 24 hours. The labelling period between 12 and 16 hours was chosen as the earliest time to add radioactive precursors in leak-free segments while falling within that interval of increased cholesterol synthesis. From experiments described below, the 4 hour labelling period proved to be sufficient to demonstrate enhanced cholesterol synthesis in arteries perfused in the absence of lipoproteins.

3.3 Perfusion Experiments

3.3.1 Scanning Electron Microspopy

3.3.1.1 Verification of Intact Endothelium

Scanning electron microscopy (SEM) was used to verify the presence or absence of endothelium in perfused arterial segments. Segments were removed as described in Methods and perfusion fixed with 2.5 % glutaraldehyde in Mallonigs' buffer for one hour at a pressure of approximately 70 mm Hg. Segments were fixed at T=0, 1 hour and 16 hours with or without endothelium. Depressurization of perfused segments promoted patchy or complete sloughing off of the endothelial lining. De-endothelialization by the method described above resulted in denudation of the luminal surface of the artery with no residual endothelial cell visible at any of the time intervals when viewed under SEM. In those segments designated as controls (with an intact endothelial lining), a uniform and continuous endothelial cell monolayer was found covering the entire lumen of the arterial segments at the start of perfusion, some sixty minutes later and at 16 hours. SEM examination of segments was conducted for five experiments or a total of thirty evaluations. Of fifteen control segments mounted for perfusion fixation, only one was found to have a ragged appearance with small denuded areas. The remainder all showed apparently healthy and confluent endothelial lining. SEM photomicrographs of segments with and without endothelium perfused for 20 minutes or 16 hours are presented in Plates 2a-2d.

Plate. 2a-2d. Scanning electon micrograph of perfused rabbit carotid artery segments. Plate (a) shows the lumen of a control segment while figure (b) depicts a similar segment with no endothelium. These segments were perfusion fixed approximately 20 minutes after dissection and prepared for SEM as described in Methods. Plates (c) and (d) represent segments with or without endothelium respectively fixed in the same manner after 16 hours of perfusion.

a

b

C

d

3.3.2 Cholesterol Regulation in Perfused Arteries With Endothelium Using Fetal Calf Serum or Lipoprotein Deficient Serum

This group of experiments was the first series to make use of arterial segments mounted in the perfusion system described in the Methods section. All segments were mounted with endothelium intact. Three segments were perfused with 10% FCS while the other three used 10 % LPDS in MEM. This experiment was conducted eight times using six segments per experiment. Twelve segments were selected at random and not evaluated for incorporation of radio-label, but instead perfusion fixed for scanning electron microscopy. Of these twelve test segments, all showed a continuous endothelial monolayer with no regions of cell loss. All other segments were evaluated for incorporation of ^{3}H-acetate into lipid and ^{35}S-methionine uptake into protein. The addition of labelled methionine provided a means of measuring tissue viability. In arteries perfused successfully for 16 hrs, labelled methionine incorporation was found to be similar between segments, regardless of the perfusate type or the presence or absence of endothelium (experiments described below) within a single experiment. The appearence of low ^{35}S-methionine incorporation was always accompanied by depressed incorporation of ^{3}H-acetate into lipid usually reflecting a technical problem with the perfusion apparatus. Thus segments perfused under less than optimal conditions were readily identified and discarded. The results presented are for experiments in which triplicate segments for a single condition were obtained unless specified otherwise. In experiments in which one or more segments were lost, tissues were analysed. While the results were discarded, they were consistent with experiments in which triplicate segments were obtained under the same conditions.

The results from one experiment in this series (Table 3) show that when segments are perfused in the absence of lipoproteins by means of LPDS, there is modest but statistically significant up-regulation of cholesterol synthesis. The average extent of this increase was similar to that seen in incubated tissues, 1,264 ± 914 cpm/mg protein in cholesterol from segments perfused with FCS compared to 3,115 ± 1,077 for that from segments perfused with LPDS, $p < 0.05$ (6 experiments). No increase in the other lipid classes was evident. As was observed in the minced tissue experiments using either FCS or LPDS, no depression in cholesterol esterification in LPDS was seen in the perfused arteries described here. Again, this may be due to the fact that the tissue in its intact form has considerable interstitial space containing residual native lipoprotein. Alternatively, it may be that esterification of cholesterol lags behind the up-regulation of de novo cholesterol synthesis temporally. It is difficult to monitor changes in esterification rate when using [3H]-acetate which incorporates not only into cholesterol but also the fatty acid pool that may be tapped for esterification. The consistent observation that de novo synthesis of cholesteol is increased by removing exogenous cholesterol in the form of LDL-cholesterol suggests that these arterial segments retain the ability to regulate cholesterol levels in a manner similar to that described in fibroblasts (Brown and Goldstein, 1973a).

Table 3. Incorporation of ^{3}H-acetate into lipid and ^{35}S-methionine into protein in perfused arteries. Segments were perfused with 10% FCS or 10% LPDS in MEM for 12 hours followed by a labelling period of 4 hours. Tissue lipids and protein were extracted, counted as described in Methods. Results are the mean ± S.D. for three segments from one experiment representative of 6 experiments.
*Significantly different from segments perfused with FCS ($p<0.05$).

	^{3}H-acetate incorporation (cpm/mg protein) FCS	LPDS
neutral lipid		
polar origin	31,760 ± 2,155	37,640 ± 6,250
mono-diglyceride	11, 704 ± 3,280	13,688 ± 1,981
cholesterol	1,418 ± 251	3,413 ± 822 *
free fatty acid	10,034 ± 2,115	14,527 ± 2,864
triglyceride	5,061 ± 1,450	7,531 ± 1,912
cholesterol ester	4,435 ± 1,450	5,236 ± 684
polar lipid		
PC	17,433 ± 3,094	20,719 ± 6,850
PE	10,857 ± 4,222	5,540 ± 3,660
SM	8,684 ± 4,994	9,814 ± 6,142
other	1,381 ± 1,277	817 ± 791

	^{35}S-methionine incorporation (cpm/mg protein) FCS	LPDS
tissue protein	8,281 ± 640	8,003 ± 791

3.4 Effect of Endothelial Removal

3.4.1 Perfusion of Arterial Segments With or Without Endothelium Using 10% Fetal Calf Serum.

This series of experiments represent the first attempt in evaluating the effect of endothelial removal and perfusion and perfusate composition. In this group, segments were also checked by means of SEM for the presence of an intact endothelium in those segments designated as control as well as demonstrating the absence of endothelium. Endothelial removal was carried out by advancing the supporting canulla toward each other and then retracting them to their initial position. The intervening segment was then remeasured to ensure that its *in vivo* length was maintained. De-endothelialization in all the experimental series described below was carried out in this manner. A typical experiment involved a preincubation of 12 hrs using MEM and 10% FCS followed by a 4 hr labelling period with 300 uCi ^{3}H-acetate and 5 uCi ^{35}S-methionine. At 16 hrs the tissue was evaluated as described in methods for radiolabel incorporation. Representative results from one experiment of this series are summarized in Table 4.

Between the two groups no difference in ^{35}S-methionine incorporation into protein nor ^{3}H-acetate uptake into neutral lipid was seen. The same was true for the separated polar lipid species with the exception of PC which was found to incorporate ^{3}H-acetate from two to three fold more in de-endothelialized segments when compared to controls (46,768 $\pm$ 11,238 cpm/mg protein in PC in controls versus 121,316 $\pm$ 49,204 cpm/mg protein in PC form de-endothelialized segments, $p < 0.05$, $n = 7$).

Table 4. Incorporation of ^{3}H-acetate into lipid and ^{35}S-methionine into protein in arteries perfused with or without endothelium. Tissues were perfused with 10% FCS for 12 hours followed by a 4 hour labelling period. Lipids and protein were extracted and counted as described in Methods. Results are the mean ± S.D. of three segments from one experiment and are typical of 7 experiments.
*Significantly different from segments perfused with endothelium ($p<0.05$).

	^{3}H-acetate incorporation (cpm/mg protein)	
	with endothelium	without endothelium
neutral lipid		
polar origin	41,257 ± 16,230	138,217 ± 30,733 *
mono-diglyceride	23,427 ± 6,358	16,865 ± 12,476
cholesterol	41,028 ± 11,964	38,821 ± 8,861
free fatty acid	22,879 ± 1,237	33,769 ± 9,486
triglyceride	39,988 ± 5,299	30,027 ± 2,862
cholesterol ester	9,017 ± 2,074	11,588 ± 3,905
polar lipid		
PC	21,737 ± 6,311	117,736 ± 65,793 *
PE	14,651 ± 6,168	10,530 ± 6,204
SM	6,741 ± 2,660	8,102 ± 4,134
other	1,852 ± 1,840	2,003 ± 1,157
	^{35}S-methionine incororation (cpm/mg protein)	
	with endothelium	without endothelium
tissue protein	18,103 ± 2,069	16,993 ± 1,083

3.4.2 Effect of Perfusion with Lipoprotein-Deficient Serum

With the observation that de-endothelialization promoted a marked increase in ^{3}H-acetate incorporation into PC, a series of experiments were conducted to determine whether this process was mediated by lipoproteins.

Perfusate was prepared using 10% lipoprotein-deficient serum and the perfusions conducted in a manner similar to that described above. Three segments served as controls with endothelium while the remaining three were de-endothelialized.

The results from eight such experiments all showed the same marked increase in ^{3}H-acetate incorporation into PC in de-endothelialized segments (48,301 $\pm$ 27,320 cpm/mg protein in PC in control segments compared to 141,282 $\pm$ 66,388 cpm/mg protein in PC from de-endothelialized segments, $p < 0.05$). Values for the other polar lipids and neutral lipids were similar to those of the controls. Table 5 shows the results from a typical experiment. ^{35}S-methionine incorporation into protein in the de-endothelialized segments also found to be similar to control values. Equivalent ^{35}S-methionine incorporation between the two groups suggests that the increase in ^{3}H-acetate uptake into PC in the de-endothelialized segments is not a reflection of a process requiring the synthesis of new protein products. From this series of experiments it was concluded that increased incorporation of ^{3}H-acetate into PC in de-endothelialized arterial segments is not dependant on the presence of serum lipoproteins.

Table 5. Incorporation of ^{3}H-acetate into lipid and ^{35}S-methionine into protein in arteries perfused with or without endothelium. Segments were perfused with 10% LPDS for 12 hours followed by a four hour labelling period. Lipid and protein were extracted as described in Methods. Results are the mean ± S.D. of three segments from one experiment and are representative of 8 experiments.
*Significantly different from segments perfused with endothelium. ($p<0.05$).

	^{3}H-acetate incorporation (cpm/mg protein)	
	with endothelium	without endothelium
neutral lipid		
polar origin	67,311 ± 9,094	178,726 ± 21,592 *
mono-diglyceride	36,686 ± 4,430	41,828 ± 9,553
cholesterol	11,050 ± 4,094	16,619 ± 4,002
free fatty acid	33,171 ± 6,198	31,604 ± 10,081
triglyceride	9,306 ± 3,965	14,807 ± 7,118
cholesteol ester	8,146 ± 4,451	8,701 ± 5,980
polar lipid		
PC	36,831 ± 5,574	107,736 ± 15,335 *
PE	29,311 ± 10,604	34,674 ± 14,389
SM	7,431 ± 4,227	11,312 ± 4,010
other	11,547 ± 8,504	10,233 ± 3,697
	^{35}S-methionine incorporation (cpm/mg protein)	
	with endothelium	without endothelium
tissue protein	42,990 ± 3,454	48,117 ± 13,314

3.4.3 Effect of Serum-Free Perfusate

The series of experiments described here were carried out to answer the question of whether any serum constituents (other than lipoproteins) were required for the observed effect to proceed. Tissues were perfused in the usual manner with the exception that serum-free perfusate was used. Using serum-free perfusate, no increase in ^{3}H-acetate into PC was seen in de-endothelialized artery segments (39,065 $\pm$ 16,577 cpm/mg protein in PC of controls compared with 42,941 $\pm$ 19,099 cpm/mg protein in PC from de-endothelialized segments, n = 4). Results from a single experiment are summarized in Table 6. From this series of experiments it was concluded that greater uptake of label into PC in the de-endothelialized segments does require the presence of some serum component other than lipoproteins to proceed.

3.4.4 Effect of Increasing Concentration of LPDS in De-endothelialized Arterial Segments

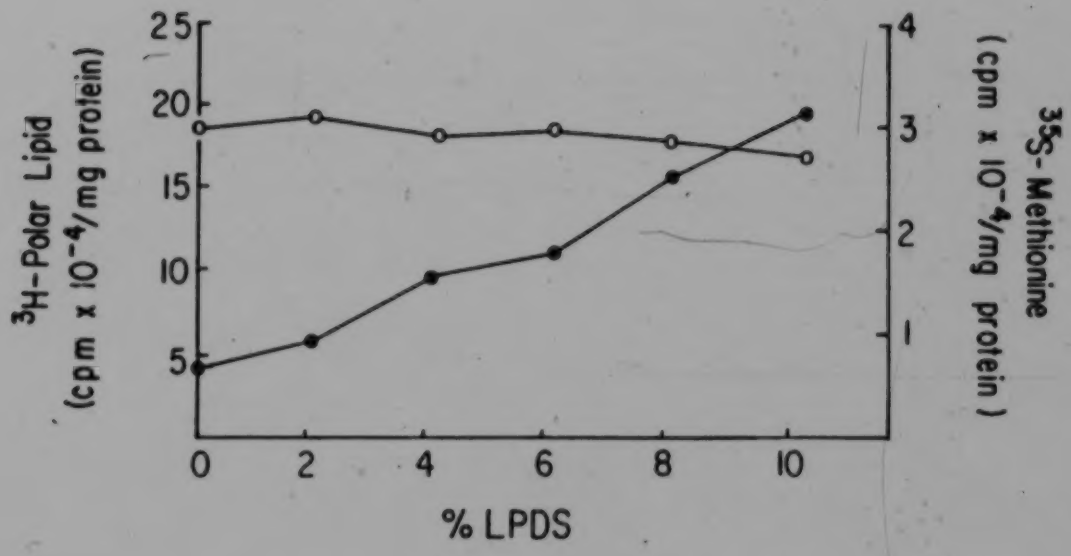
Perfusion of de-endothelialized segments in the presence of serum (either FCS or LPDS) promoted an increase in ^{3}H-acetate into PC. By removing serum from the perfusate no such increase was observed. To determine if the extent of this increase was related to the serum concentration, de-endothelialized segments were perfused with increasing concentration of LPDS. The results from this experiment is presented in Figure 6. Each point corresponding to a particular serum concentration represents ^{3}H-acetate incorporation into PC from a single tissue sample differing from the majority of results presented (the mean of triplicate samples). Since considerable variability was

Table 6. Incorporation of ^{3}H-acetate into lipid and ^{35}S-methionine into protein from arteries perfused with or without endothelium. Segments were perfused serum-free media for 12 hours followed by a 4 hour labelling period. Lipid and protein were extracted as described in Methods. Results are the mean ± S.D. of three segments from one experiment and are representative of 4 experiments.

	^{3}H-acetate incorporation (cpm/mg protein) with endothelium	without endothelium
neutral lipid		
polar origin	44,424 ± 2,279	47,764 ± 3,492
mono-diglyceride	19,460 ± 6,490	22,843 ± 4,871
cholesterol	7,610 ± 2,859	9,051 ± 3,750
free fatty acid	18,616 ± 5,768	14,931 ± 4,450
triglyceride	9,758 ± 2,051	6,705 ± 3,955
cholesterol ester	3,961 ± 1,965	5,058 ± 2,183
polar lipid		
PC	22,375 ± 6,812	19,044 ± 2,872
PE	12,687 ± 4,626	15,465 ± 6,054
SM	9,804 ± 7,936	4,823 ± 2,485
other	3,854 ± 2,367	2,239 ± 860

	^{35}S-methionine incorporation (cpm/mg protein) with endothelium	without endothelium
tissue protein	11,164 ± 1,495	13,620 ± 2,956

Figure 6. Effect of increasing serum concentration on ^{3}H-acetate incorporation into PC (solid circles) and ^{35}S-methionine uptake into protein (open circles) in six de-endothelialized segments. Arteries were perfused with MEM plus varying concentration of LPDS (0-10%). Incorporation results represent single samples from individual arteries corresponding to a given LPDS concentration.



observed between different animals, effort was made in this experiment to use six segments from one animal. Incorporation of ^{35}S-methionine into protein from these segments is also included in Figure 6 and shows that in the presence of increasing serum concentration protein synthesis remains similar in all six segments. The extent of incorporation of ^{3}H-acetate by PC however increased in a dosage-dependent fashion. The other polar and neutral lipids were also evaluated for ^{3}H-acetate uptake and no difference between the two groups was evident (data not presented).

3.4.5 Serum Factor

De-endothelialization is observed to promote an increase in the incorporation of ^{3}H-acetate into PC although no increase in PC content is seen. This turnover process is apparent only when arterial segments are perfused with media supplemented with whole serum but also proceeds equally well if LPDS is used. No turnover occurs when segments are perfused with serum-free media. Thus, turnover of PC due to de-endothelialization demonstrates a requirement of a serum component other than lipoprotein.

3.4.5.1 Ammonium Sulphate Fractionation

Five subfractions of LPDS were produced using the ammonium sulphate precipitation procedure described in Methods. The amount of measured protein in each fraction is presented in Table 7. Each fraction was tested for ability to promote increased PC turnover in de-endothelialized segments in perfusion. The results from this assay are summarized in Table 8. Incorporation for segment 1 perfused with complete LPDS was seen to be 98,344 cpm/mg protein in PC and comparable results were also observed in segments 2 and 3 perfused with the first

Table 7. Protein content of LPDS subfractions. Increasing concentrations of ammonium sulphate were used to successively precipitate protein from LPDS. Protein content was determined for each fraction as described in Methods. Results represent the protein content of indvidual LPDS subfractions.

LPDS FRACTION	ammonium sulphate mg/ml	protein mg/ml
1	0	46.1
2	60	41.3
3	120	28.6
4	180	19.9
5	240	12.3
6	300	0.0

Table 8. Bioassay of LPDS-ammonium sulphate precipitated subfractions. Protein reduced LPDS fractions were perfused through de-endothelialized arteries for 16 hours with ^{3}H-acetate and ^{35}S-methionine for the last 4 hours. Tissue lipid and protein were extracted and incorporation of labled acetate into PC determined as described in Methods. Results represent single values for uptake of ^{3}H-acetate by PC corresponding to a given LPDS fraction for 6 arterial segments constituting one experiment. This experiment is typical of three experiments using the same LPDS sub-fractions.

		^{3}H-acetate into PC	^{35}S-methionine into protein
Perfusate fraction			
	1	98,344	19,602
	2	58,391	23,198
	3	76,424	18,509
	4	19,051	16,431
	5	11,390	20,199
protein-free	6	21,453	17,640

and second subfractions respectively. PC uptake of ^{3}H-acetate in segments 4 and 5 however were seen to be 1/3 to 1/4 of that of the control segment but in line with segment 6 perfused with protein-free serum as demonstrated in the previous table. Thus it would appear that the protein responsible for the increase in PC turnover remains in the first and second LPDS fractions but is lost in the last three fractions. Precipitated protein from fraction 4, 5 and 6 were resuspended by extensive dialysis to remove ammonium sulphate and again checked for bioactivity in de-endothelialized arteries. Table 9 shows the enhanced PC turnover in segment 1 perfused with untreated LPDS. Segments 5 and 6 were perfused with protein free-ammonium sulphate precipitated LPDS used in the previous experiment showing PC turnover as reflected by incorporation of ^{3}H-acetate to be less than half that seen in the first segment. The second, third and forth segments, perfused with MEM containing resuspended protein precipitated from fractions 4, 5 and 6. These showed uptake of labelled acetate into PC to an extent similar to that seen in the remaining two arteries perfused with serum-free media. This would suggest that the protein factor promoting greater PC turnover in de-endothelialized arteries may not readily be recovered by this method after precipitation or may be altered.

3.4.5.2 Electrophoresis

Protein from LPDS, active ammonium sulphate fractions 1, 2, and 3 and inactive fractions 4, 5, and 6 were seperared by SDS-Polyacrylamide Gel Electrophoresis (Laemmli, 1970). Samples were run on 8%

Table 9. Bioassay of protein precipitated from LPDS. Ammonium sulphate precipitated protein was resuspended by dialysis as described in Methods and perfused through de-endothelialized arteries for 16 hours. ^{3}H-acetate and ^{35}S-methionine were included in perfusate for the last 4 hours. Results represent the incorporation of labled acetate in PC for a single arterial segments corresponding to one resuspended protein fraction. These data are typical of 4 experiments using the same resuspended protein fractions.

		cpm/mg protein	
Perfusate fraction with resuspended protein.		^{3}H-acetate into PC	^{35}S-methionine into protein
Untreated LPDS		71,699	16,094
Resuspended Fraction	#4	22,905	12,189
	#5	27,407	17,641
	#6	30,611	13,803
Serum-free		34,370	15,391
Serum-free		24,391	19,011

acrylamide gels under reducing or non-reducing conditions. No unique band(s) were detected in LPDS or the active fractions. A prominent albumin band was apparent in all fractions. In subsequent runs, the samples were pretreated with Blue sepharose. This removed 50-75% of the albumin but again no unique protein bands were detected in the active fractions compared to the separated inactive fractions.

3.5 Barrier experiments

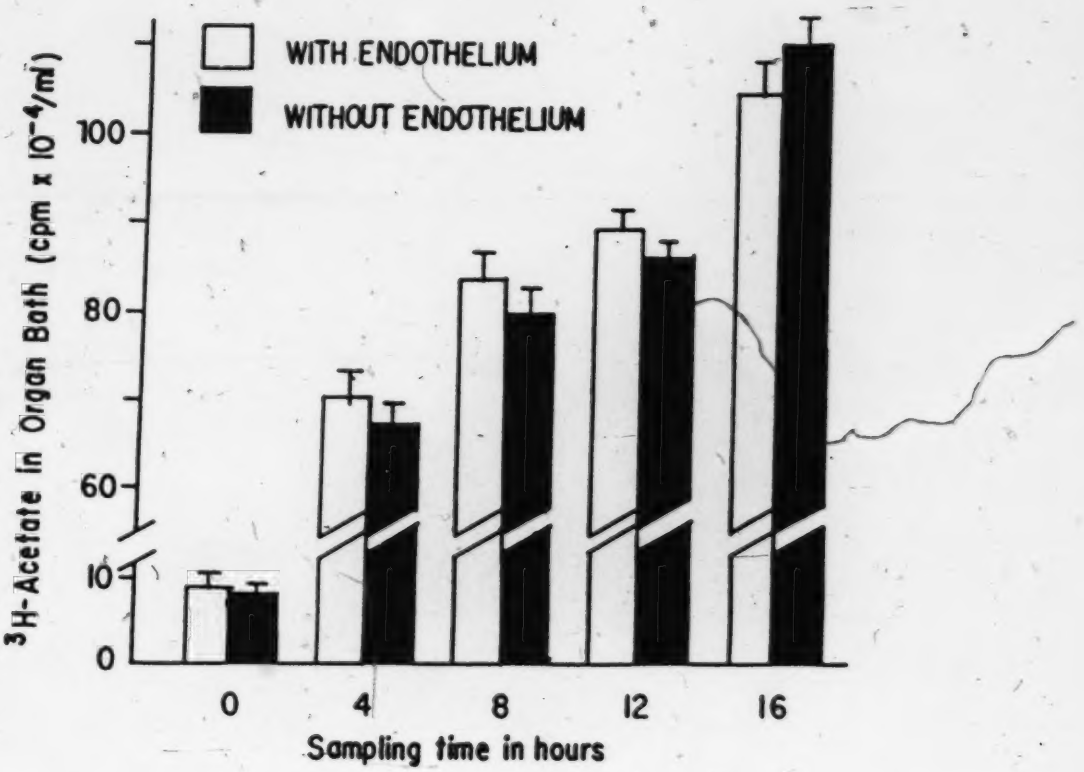
3.5.1 Effect of De-endothelialization On ^{3}H-Acetate Flux Across the Across the Artery Wall

An important physiological role played by the endothelium is to provide a non-thrombogenic surface for blood containment. The intact endothelium also acts as a barrier the function of which is not well understood. The observation has been made that de-endothelialization promotes greater ^{3}H-acetate uptake in PC in the segments perfused with FCS or LPDS. This increase could be due to the fact that endothelial removal allows greater flux or accessibility of the labelled precursor. This might artificially give rise to PC of greater relative specific activity. In this event the observation of greater ^{3}H-acetate incorporation into PC in de-endothelialized segments would not be a true reflection of any biological response. In order to rule out the possibility of any such barrier process operating the following experiments were carried out.

3.5.1.2 Movement of ^{3}H-Acetate From the Artery Lumen into the Bathing Medium

Perfusate containing 10% FCS was used in these experiments as well as in the next series described below. 300 uCi of ^{3}H-acetate was added to the perfusion media immediately after the arterial segments were placed into the perfusion apparatus. Since the flux of radioactivity is also a function of the length of tissue perfused, segments were measured carefully and supporting perfusion holders adjusted such that 1.25 cm lengths were suspended between the cannulae in all six preparations. At four hour intervals, duplicate aliquots of the bathing buffer were removed, added to scintillant cocktail and counted to determine the eflux of ^{3}H-acetate from the lumen, across the artery wall into the containment vessels. The recovery of radioactivity was then calculated on a per ml basis and presented in Figure 7. The movement of ^{3}H-acetate was found to be similar across both the control and de-endothelialized segments and increased in a linear fashion when measured at subsequent time intervals. At eight hours the movement of radioactivity appeared to be slightly greater in those segments with endothelium although this increase was not significant. Incorporation of ^{3}H-acetate into neutral and polar lipid with the exception of PC were all observed to be similar in the control and de-endothelialized groups. The uptake of ^{3}H-acetate into PC in the de-endothelialized segments was observed to be 26,446 $\pm$ 3,825 cpm/mg protein versus
5,773 $\pm$ 1,165 cpm/mg protein seen in PC of the controls (significantly different $p < 0.05$). The appearance of radioactivity in the bathing

Figure 7. Monitoring of tissue bathing buffer for the appearance of ^{3}H-acetate traversing the artery wall in perfused segments with or without endothelium. Labelled acetate was added to the perfusate (10% FCS in MEM) at the start of perfusion and the bathing buffer measured for radioactivity at 4 hour intervals for 16 hours as described in Methods. Results are the mean ± S.E. of three tissue bath vessels containing individual segments.



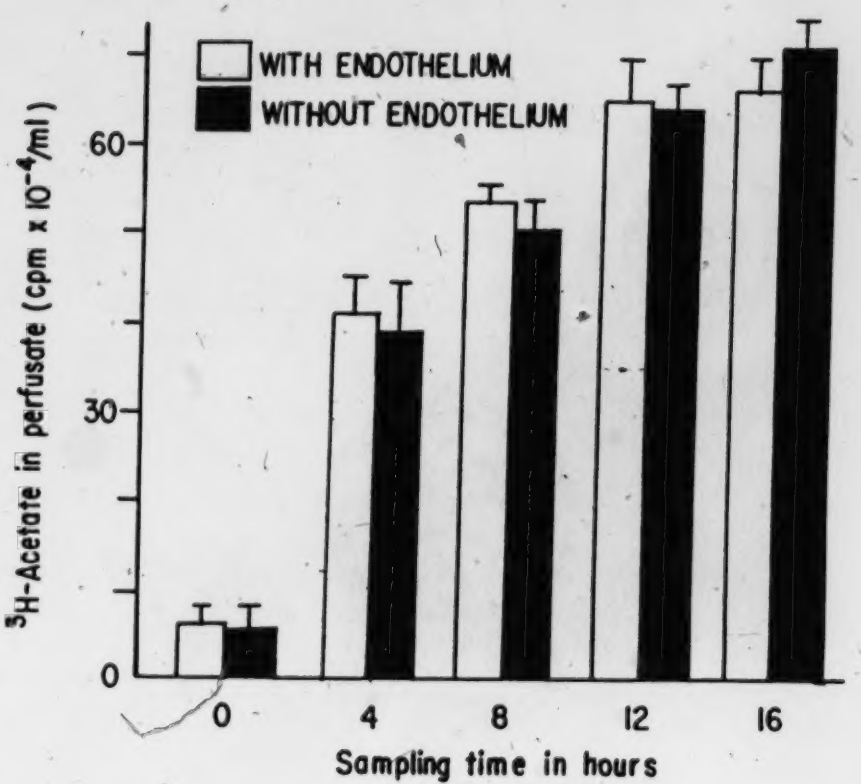
medium was evaluated in two experiments with similar out come. From these data it was concluded that the rate of efflux of ^{3}H-acetate was similar in all segments with or without endothelium.

3.5.1.3 Movement of ^{3}H-Acetate From the Bathing Medium into the Perfusate

From the previous experiments the appearance of radioactivity in the bathing was detected to the same extent in baths holding segments with or without endothelium. If however de-endothelialization does allow greater flux of labled acetate but there exists a zone within the artery wall that coincidently limits the rate of its efflux, then the observation of enhanced uptake of ^{3}H-acetate into PC may still be due to greater accessibility of precursor. The question of accessibility is not generally a concern in studies using cell monolayers in tissue culture. However a segment of artery may be many hundreds of cells thick and contains matrix materials and elastic laminae. This structural complexity could well present a limiting barrier to acetate flux.

The experimental procedure was modified to test the possibility of such a barrier phenomenon. ^{3}H-acetate was added to the bathing medium instead of the perfusate. The containment vessels were miniaturized to present comparable levels of radiolabelled precursor to the adventitial side of the segments. The perfusate was sampled at four hour intervals for the appearence of radioactivity and the findings are presented in Figure 8. The rate of ^{3}H-movement across the arterial segments was again found to be similar in both control and

Figure 8. Monitoring of tissue perfusate for the appearance of ^{3}H-acetate crossing the artery wall in perfused segments with or without endothelium. Labelled acetate was added to the bathing buffer at the start of perfusion and perfusion media (10% FCS in MEM) measured for radioactivity at 4 hour intervals for 16 hours as described in Methods. Results are the mean ± S.E. from three reservoirs used to perfuse segments with or without endothelium.



de-endothelialized tissues. The values for incorporation of ^{3}H-acetate into lipid shows greater uptake only in PC from de-endothelialized segments while values for the other lipid species are similar in both groups (12,124 ± 4,514 cpm/mg protein in PC in control segments versus 44,734 ± 5,570 cpm/mg protein in PC from de-endothelialized segments, $p < 0.01$). If a barrier was present in the artery wall at a point external to the endothelium promoting a region of greater ^{3}H-acetate concentration, then presentation of label from the adventitial side should ensure similar rates of ^{3}H-acetate influx. At the same time, no increase in ^{3}H-PC should be observed in the de-endothelialized arteries. The results presented here suggest that such a process is not occuring with the observation of a three-fold increase in ^{3}H-acetate incorporation into PC of de-endothelialized segments. These findings coupled with those from the previous experiment led to the conclusion that the flux of label is similar across the artery wall regardless of whether there is an endothelium. With endothelial removal there would appear to be an enhancement in the rate of ^{3}H-acetate incorporation into PC due only to endothelial loss and not preferential accessibility of labelled presursor. This conclusion is supported by the observation that in the 8 other lipids classes evaluated no differences between control and de-endothelialized segments were observed.

3.6 Time Interval Experiments

3.6.1 Short Term Perfusion: 0-2, 0-4, 0-8, 0-12 Hours

In the previous experiments, ^{3}H-acetate was added to perfusate after 12 hours of perfusion. The observation of greater uptake of ^{3}H-acetate into PC in de-endothelialized segments reflects some process occuring from 12 to 16 hours. Short-term perfusion experiments were conducted to determine the time course of the incorporation of acetate into PC in de-endothlialized arterial segments.

The experimental routine was modified by adding ^{3}H-acetate to the perfused segments immediately after de-endothelialization and the segments collected after a total of 2 hours. Separate experiments were conducted to evaluate the perfusion intervals 0 to 4, 0 to 8 and 0 to 12 hours. For perfusions totalling 8 or 12 hours, ^{3}H-acetate was added to the perfusate for the last 4 hours. For the 0 to 4 hour time interval, labelled acetate was added for the last two hours. Duplicate experiments were conducted for each interval and the results summarized in Table 10. Over all the time periods examined, significantly enhanced incorporation of ^{3}H-acetate into PC was observed in de-endothelialized segments relative to that obseerved in the controls. No appreciable difference in uptake of ^{3}H-acetate into the remaining phospholipids or in any of the neutral lipids was observed between the two groups of segments.

These observations suggest that de-endothelialization promotes greater uptake of labelled acetate into PC almost immediately and that this increase persists over the entire 16 hour period.

Table 10. Incorporation of ^{3}H-acetate into phosphatidylcholine in arterial segments perfused with 10% LPDS with or without endothelium for four time intervals (one experiment/interval). Phosphatidylcholine was extracted as described in Methods. Results are the mean ± S.D. of triplicate samples.
*Signifiantly different from PC from segments with endothelium ($p<0.05$).

	^{3}H-acetate incorporation into PC (cpm/mg protein)	
	with endothelium	without endothelium
Perfusion interval		
0-2 hours	28,101 ± 7,562	57,822 ± 12,431 *
0-4 hours	24,375 ± 11,897	46,637 ± 10,804 *
0-8 hours	57,304 ± 6,073	88,106 ± 18,535 *
0-12 hours	36,656 ± 4,880	77,771 ± 12,173 *

3.7 Phosphlipid Mass Determination

For the experiments in which an increase in ^{3}H-acetate into PC was observed (de-endothelialized using FCS or LPDS), individual phospholipid content was determined. Lipid phosphorous was measured in individual phospholipid species from both control and de-endothelialized arteries. These results are summarized in Table 8 for a single experiment in which segments were perfused with 10% LPDS. Phospholipid content is presented in the form of ug phospholipid species normalized to tissue protein and also in cpm ^{3}H-phospholipid/ug phospholipid (specific radioactivity). In terms of lipid mass there are essentially the same amounts of PC in both control and de-endothelialized segments (126.5 $\pm$ 14 ug PC/mg protein in controls compared with 131.1 $\pm$ 17 ug PC/mg protein from de-endothelialized segments, n = 8). The results for individual phospholipid species from one experiment are shown in Table 11. The observed increase in ^{3}H-acetate incorporation in PC from de-endothelialized arteries is evident only in terms of increased specific activity of PC, 1,239 $\pm$ 336 cpm/ug PC and 435.2 $\pm$ 222 cpm/ug PC respectively ($p < 0.05$, n = 8). The demonstration of greater incorporation of ^{3}H-acetate into PC in the absence of any net increase in PC mass suggests that de-endothelialization promotes greater PC turnover.

Table 11. Determination of mass and specific activity of major phospholipid species from arterial segments with or without endothelium. Segments were perfused with 10% LPDS in MEM for 12 hours followed by a 4 hour labelling period with ^{3}H-acetate and ^{35}S-methionine. Lipid was extracted, separated by TLC and phosphate content and incorporation of ^{3}H-acetate into phospholipid species was determined as described in methods. Results are the mean ± S.D. of triplicate samples and are typical of 8 experiments using LPDS supplemented perfusate.

	ug phospholipid/mg protein	
	with endothelium	without endothelium
PC	136.6 ± 14.9	131.8 ± 18
PE	46.3 ± 14.6	52.1 ± 16.3
SM	58.4 ± 10.1	56.2 ± 8.1

	^{3}H-phospholipid/ug phospholipid	
	with endothelium	without endothelium
PC	494 ± 166	1,364 ± 291 *
PE	637 ± 123	667 ± 168
SM	128 ± 41	203 ± 66

3.8 Pulse-Chase Verification of Increased Turnover in PC Due to Endothelial Removal

3.8.1 Preincubation with ^{3}H-acetate Before Endothelial Removal

To determine whether increased ^{3}H-acetate incorporation into PC was a reflection of PC turnover, the following experiment was conducted. Six segments were mounted into the perfusion apparatus and verified to be leak-free. ^{3}H-acetate was added at a concentration of 300 uCi per segments at T_o. At the 8 hour point, the perfusate was changed several times to remove the labelled acetate and three of the six segments were de-endothelialized. Approximately 30 minutes later, one control and one de-endothelialized segment were collected. A second control and de-endothelialized segment were collected at 2 hours and the remaining pair removed at a point 4 hours after endothelial removal.

The rationale behind this experiment stems from the following considerations. The incorporation of ^{3}H-acetate into PC is most likely in the form of labelled fatty acids since the remainder of the molecule is not derived from acetate. Choline is an essential dietary requirement and and the phospholipid glycerol backbone originates from glucose. The appearance in ^{3}H-in PC is primarily a reflection of label located in the fatty acyl chains of PC.

Perfusion of segments with ^{3}H-acetate was carried out to pre-label PC. If de-endothelialization does promote greater turnover of fatty acyl portions of PC, then endothelial removal coupled with the

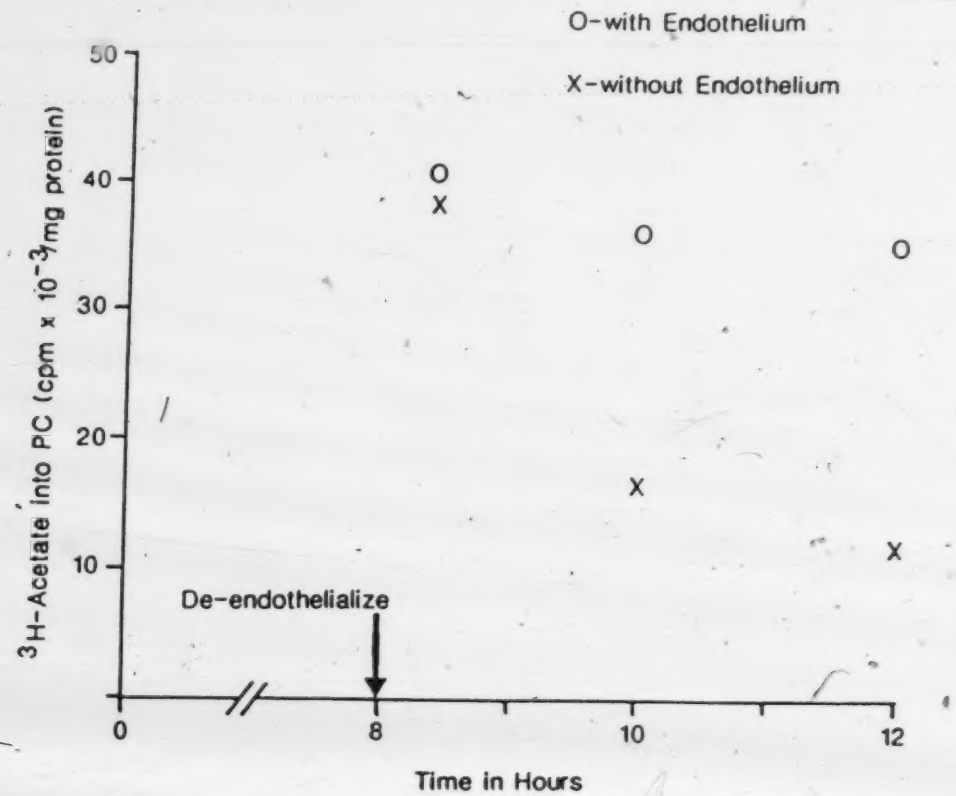
replacement of perfusate with fresh unlabelled media should cause a more rapid loss of radioactivity from the PC molecule and this decrease should be more evident over time.

This was shown to be the case with the extent of ^{3}H remaining in PC presented in Figure 9. For PC from segments collected at 30 minutes after endothelial removal, a slight drop in radioactivity was observed in PC from de-endothelialized segment relative to the control. At the 2 and 4 hour intervals however, a marked and increasing loss of ^{3}H in PC was seen. The disappearance of radioactivity from PC in the controls was also observed since it too is turning over at some basal rate but not to the extent as that seen for the de-endothelialized counterpart collected at the same time after endothelial removal. Thus it can be concluded from this group of experiments that endothelial removal promotes increased turnover of the fatty acid component of PC. The results presented here represent single samples, one determination per segment or a total of six points. This experiment was conducted 3 times showing similar results.

3.8.1.1 Analysis of Perfusate for Labelled Product

Results from the previous experiment suggest that increased turnover as indicated by a more rapid loss of radioactivity in PC reflects the liberation of a newly labelled component of PC. Assuming that this released PC constituent was fatty acid, then it may be detected in the total fatty acid extracted from the tissue. If subsequently reincorporated into other cellular lipid species its

Figure 9. Demonstration of increased turnover of PC in de-endothelialized arterial segments. Six segments were perfused with 10% LPDS in MEM plus ^{3}H-acetate. At 8 hours, perfusate was replenished several times with unlabelled media and three segments were de-endothelialized as described in Methods. Thirty minutes later a control and de-endothelialized segment were collected and again 2 and 4 hours later. Results represent single samples for ^{3}H-acetate incorporation into PC from three control or three de-endothelialized segments collected at the above times.



appearence may also be apparent. As is observed in tissue culture, the released product may be detectable in the media, in this case the perfusate or bathing buffer. Attempts to demonstrate the released labelled PC component by evaluating tissue lipid, bathing buffer and perfusate were not successful. Demonstration of labelled fatty acid in the perfusate may have been obscured by high residual ^{3}H-acetate levels. No apparent increase in radioactvity could be seen in any of these components between control and de-endothelialized segments.

3.9 Demonstration of Greater Fatty Acid Turnover in De-endothelialized Segments

3.9.1 Double Label Perfusion Using ^{14}C-Acetate and ^{3}H-Choline

The observations to this point suggest that endothelial removal promotes an increase in PC with no increase in the net amount of PC. This effect demonstrates a requirement for serum but not lipoproteins. With the use of ^{3}H-acetate, the data suggest that the observed turnover process minimally involves the fatty acyl portions of the PC molecule. However using this labelled precursor does not rule out the possibility of turnover by other parts or the entire PC molecule.

To address the possibility of other portion(s) of PC participating in this process of turnover, experiments were conducted using 25 uCi ^{3}H-choline (in addition to ^{14}C-acetate) which permits labelling of the head group of PC. Segments were perfused with or without endothelium for an initial 12 hours using media supplemented with 10% LPDS and both labelled precursors added for 4 hours or a total

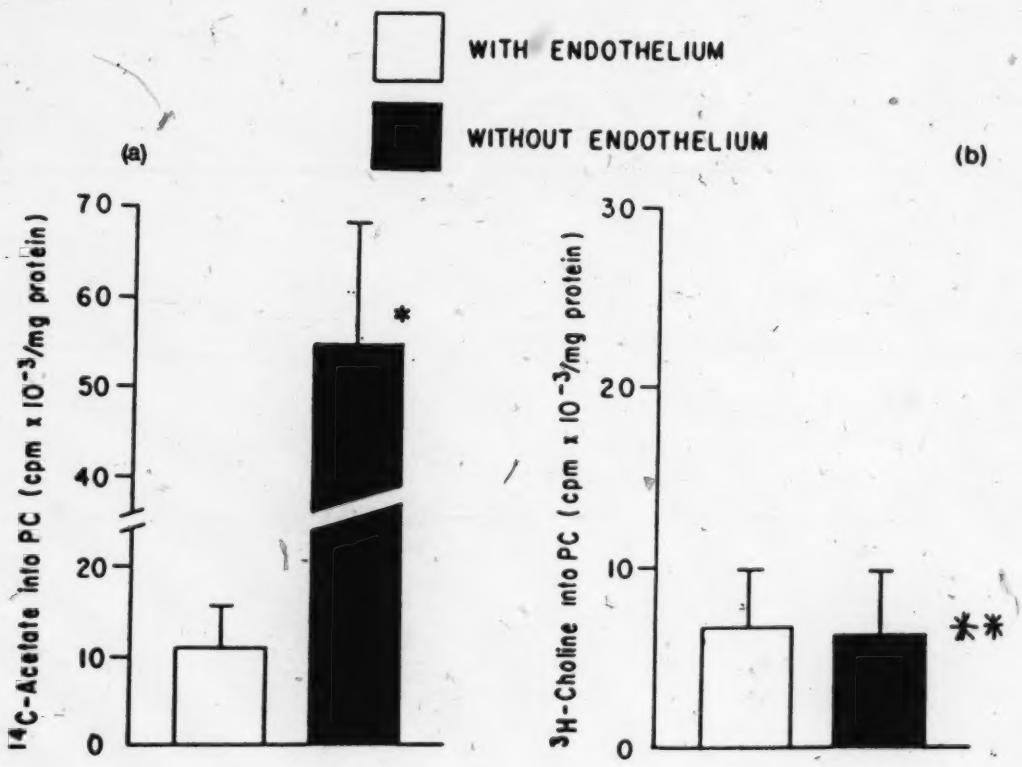
perfusion time of 16 hours. Tissue was extracted and lipids separated by TLC as described in Methods. Isolated PC was evaluated for incorporation of radiolabelled choline and acetate. Results from this experiment are presented in Figure 10.

The previously made observation of greater labelled acetate incorporation in PC from de-endothelialized segments is readily apparent. However, the extent of ^{3}H-choline uptake into PC in de-endothelialized segments parallels that observed in PC of the control segments. In addition, remaining phospholipids, neutral lipids and ^{35}S-methionine incorporation into protein were all found to be similar between the two groups (data not shown). The observation of a lack of increase in ^{3}H-choline content in PC from de-endothelialized arteries but a marked increase in ^{14}C-acetate in the same phospholipid supports the tentative conclusion that enhancement of PC turnover involves primarily the fatty acyl constituents of the molecule.

3.10 Fatty Acid Composition of Phosphatidylcholine.

Changes in fatty acid composition through a process of turnover are reported widely in the literature (Arthur and Choy, 1984, Chakravarthy et al, 1986). Many studies show preferential deacylation or reacylation involving specific fatty acid species. The use of gas-liquid chromatography makes the determination of fatty acid species relatively straight forward.

Figure 10. Incorporation of ^{3}H-choline and ^{14}C-acetate into PC in perfused arterial segments with or without endothelium. Tissues were perfused with 10% LPDS in MEM for 12 hours followed by a 4 hour labelling period. Data represents the mean ± S.E. from 4 experiments.
*Significantly different from ^{14}C-PC of controls.
**Not significantly different from ^{3}H-PC of controls.



Phospholipid from control and de-endothelialized artery segments was evaluated for total fatty acid content. Phospholipid species were separated from lipid extracts and transmethylated. Fatty acid composition was determined for PI, PE, PS, SM (data not presented) and the fatty acids detected in PC are presented in Table 12. No species specific loss or gain is apparent in the fatty acid profile of PC from de-endothelialized arterial segments. Arachidonic acid content was found to be approximately 6% of the total and the remaining fatty acid species were also similar between the two groups. No conclusion can be made from these observations, however it is worth noting that such analysis represents the compositional evaluation of fatty acids residing at both the sn-1 and sn-2 positions on the PC molecule. If a preferential reshuffling of a particular fatty acid were occuring at only one location on the molecule, this might be less apparent by evaluating the entire fatty acid complement of PC.

3.10.1 Phospholipase A2 hydrolysis of Phosphatidylcholine

De-endothelialization was found to promote an increase in PC acyl chain turnover. This observation gives no indication of whether this process is occuring at the sn-1 or sn-2 position of the PC molecule. This is important in considering possible changes in fatty acid composition. Equal increase in incorporation of labelled acetate into both sn-1 and sn-2 fatty acids would suggest greater <u>de novo</u> PC synthesis and degradation. However, previous results from experiments using ^{3}H-choline are not in support of such a process. Alternatively, a deacylation-reacylation process occuring at one fatty acid position would be consistent with the experimental observations.

Table 12. Total fatty acid composition of phosphatidylcholine form arterial segments with or without enothelium perfused with 10% LPDS in MEM. Segments were perfused for 16 hours, phosphatidylcholine extracted and transmethylated for gas-liquid chromatography as described in Methods. Results are presented as percent of total fatty acid detected and are the means ± S.D. of triplicate samples.

fatty acid species (% of total)	with endothelium	without endothelium
16:0	15.8 ± 2.6	15.6 ± 3.3
16:1	0.94 ± 0.40	0.54 ± 0.17
18:0	12.4 ± 2.22	13.8 ± 2.09
18.1	18.8 ± 2.29	19.2 ± 2.01
18:2	1.48 ± 0.49	1.23 ± 0.71
20:0	0.48 ± 0.15	0.77 ± 0.23
20:4	5.56 ± 0.76	6.10 ± 0.55
22:0	4.56 ± 0.72	5.02 ± 1.08

Position specific phospholipases are useful tools since they provide for site-specific hydrolysis of substrate phospholipid. Phospholipase A2 is particularily well suited for use in the study described here since this enzyme preferentially releases the fatty acyl chain residing at the sn-2 or second carbon on the glycerol back bone of PC.

3.10.11 Phospholipase A2 Hydrolysis of Phosphatidylcholine: Gas Chromatographic Analysis of sn-2 Fatty Acid and Residual Lysophosphatidylcholine

Phosphatidylcholine separated from control and de-endothelialized arteries from 5 LPDS and 3 FCS experiments was subject to phospholipase A2 hydrolysis. The efficiency of the hydrolysis process was evaluated by first demonstrating essentially total cleavage of fatty acid (sn-2) from prepared DPPC- labelled in both fatty acid chains as described in Methods. From the PC-phospholipase reaction mixture, product sn-2 fatty acid released, residual lysoPC and intact PC were separated. The results of the standardization procedure for hydrolysis of ^{3}H-DPPC are summarized in Table 13. Products generated by hydrolysis were identified on TLC plates by co-migration with known fatty acid, lysoPC and PC standards run on adjacent lanes. Table 13 shows only slight radioactivity remains on the TLC plate in the location corresponding to PC. Visualization of lipid under iodine vapor was also negligible in

Table 13. Hydrolysis of ^{3}H-dipalmitoyl phosphatidylcholine labelled in both fatty acyl chains with phospholipase A2. ^{3}H-dipalmitoyl phosphatidylcholine was hydrolysed with phospholipase A2 for 90 minutes. Products were seperated and counted as described in Methods. Results are the mean ± S.D. of triplicate samples and are typical of four such procedures.

	^{3}H detected	
	+PLA2	-PLA2
DPPC (initial)	20,442 ± 3,198	21,414 ± 1,508
DPPC (residual)	1,068 ± 199	20,020 ± 254
lysoPC	8,610 ± 934	311 ± 181
sn-2 fatty acid	9,083 ± 558	398 ± 197

this region of the TLC plate. The extent of iodine staining in the position corresponding to fatty acid and lysoPC were marked and comparable to the eye. Radioactivity was detected in approximately equal amounts in these latter two spots on the TLC plates indicating that essentially all the DPPC had been broken down to the lyso form and fatty acid from the sn-2 position. Phospholipases and their use as tools for the study of lipid composition has been extensively reviewed by Van den Bosch, (1980). Cautious use of phospholipase A2 is indicated due to the tendancy of acyl chain migration from the one position to the two position occuring in the product lyso phospholipid. By using 0.1M borate buffer in the reaction mixture, acyl chain migration is prevented.

PC from control and de-endothelialized arterial segments were subject to the same hydrolysis procedure with phosphlipase A2 from the first experiment using LPDS supplemented perfusate. Fatty acid, lysoPC and PC remaining were separated from the reaction mixture, constituent fatty acids transmethylated and evaluated using GC. The major fatty acid species detected from one such analysis is presented in Table 14. No fatty acids were detected from extracted TLC spots corresponding to intact PC. Fatty acid content from lysoPC showed a greater relative amount of the major saturated fatty acid species than that observed in the sn-2 fatty acid profile. Greater occurence of unsaturated species at the sn-2 position is typical of many phospholipid species (Vance,1985). However, fatty acid composition of lysoPC and fatty acid released from the sn-2 position from PC of de-endothelialized artery segments very closely resembled that of the control counterparts. No species specific process of deacylation or reacylation was evident in this analysis.

Table 14. Fatty acid composition of lyso PC and released sn-2 fatty acid of PC. Segments were perfused with 10% LPDS for 16 hours with or without endothelium and PC extracted, hydrolysed with phospholipase A2 and product fatty acids transmethylated for GC analysis as described in Methods. Results are mean ± S.D. of triplicate samples. Data is expressed as percent of total fatty acid detected from a single experiment and is representative of 8 evaluations on PC from segments perfused with LPDS.

	lysoPC fatty acid composition (% of total)	
	with endothelium	without endothelium
Fatty acid species		
16:0	16.6 ± 2.55	15.9 ± 1.12
16:1	0.6 ± 0.14	0.5 ± 0.17
18:0	14.7 ± 2.28	15.7 ± 1.72
18:1	19.9 ± 1.70	21.1 ± 1.87
18:2	1.6 ± 0.31	1.3 ± 0.54
20:0	2.7 ± 1.1	1.8 ± 0.5
20:4	2.3 ± 0.81	1.9 ± 1.86
22:0	2.1 ± 0.68	1.7 ± 1.24

	sn-2 fatty acid composition (% of total)	
	with endothelium	without endothelium
16:0	20.6 ± 1.98	20.8 ± 1.72
16:1	1.1 ± 0.48	1.2 ± 0.13
18:0	17.6 ± 1.17	17.2 ± 1.36
18:1	23.1 ± 1.69	22.6 ± 2.48
18:2	1.1 ± 0.80	1.6 ± 0.91
20:0	0.35 ± 0.07	0.41 ± 0.11
20:4	6.3 ± 0.17	5.8 ± 0.50
22:0	3.6 ± 0.72	2.9 ± 0.39

3.10.12 Phospholipase A2 Hydrolysis of Phosphatidylcholine: ^{3}H-acetate Incorporation into Released Fatty Acid and Residual Lysophosphatidylcholine

The GC analysis conducted on PC from control and de-endothelialized arterial segments and products released through hydrolysis with phospholipase A2 were also examined for incorporation of ^{3}H-acetate. Half of the starting PC was used for the GC analysis while the remainder was analysed for radioactivity. This PC was resuspended in chloroform and halved again. One half was counted for total uptake of ^{3}H-acetate while the remainder was hydrolysed with phospholipase A2. Total PC radioactivity, and that detected in released fatty acid and lyso PC are summarized in Table 15 for one experiment. PC phosphorous was measured to ensure the same amounts of starting material before hydrolysis. Although the initial mass of PC is similar between that from control or de-endothelialized segments, radioactivity is approximately two-to three fold greater in PC from segments in which the endothelium had been removed. LysoPC from both groups is also seen to contain comparable radioactivity. However, the fatty acid released by phospholipase A2 hydrolysis of PC from de-endothelialized segments is observed to contain in excess of two times the radioactivity of that of the controls. Mean values for released sn-2 fatty acid from PC were 33,508 $\pm$ 18,870 cpm/mg protein in controls compared with 73,280 $\pm$ 22,465 cpm/mg protein from de-endothelialized segments ($p < 0.05$, $n = 5$). Although no compositional change is apparent with respect to fatty acid component of PC, it can be concluded that the deacylation-reacylation or enhanced turnover is specifically occuring at the sn-2 position on the PC molecule.

Table 15. ^{3}H-acetate incorporation in lysoPC and sn-2 fatty acid from PC hydrolysed with phospholipase A2. PC was extracted from arterial segments that had been perfused with or without endothelium for 16 hours using LPDS supplemented media. Labelled acetate was presented to the tissues for the last four hours of the perfusion peiod. PC was seperated on TLC, hydrolysed with phospholipase A2 and products counted as described in Methods. Results are the mean ± S.D. of triplicate samples and are representative of 5 experiments analysed. *Significantly different from sn-2 fatty acid released from PC extracted from segments with endothelium ($p < 0.01$).

	^{3}H detected	
	with endothelium	without endothelium
PC (cpm/mg protein)	26,845 ± 3,762	66,897 ± 5,481
PC (residual)	1,403 ± 496	2,663 ± 1,355
lysoPC	9,038 ± 2,012	14,619 ± 4,035
fatty acid (sn-2)	16,240 ± 3,978	48,312 ± 8,649 *

3.11 Effect of endothelial cell conditioned perfusate on PC turnover in de-endothelialized arterial segments

Many cells maintained in culture display the ability to condition the medium in which they grow. Insight into complex cell interaction can be investigated *in vitro* by exposing fresh cultured cells to media that has been previously conditioned in this way.

The perfusion system described here represents a crude *in vitro* model for the study of the the artery preserving some of its *in vivo* features. Though the question of changes in lipid metabloism in this system has been examined to some extent, the issue of the endothelium mediating an inhibition of turnover in the control segments was not addressed. The working assumption was that the intact endothelium functions in some way to inhibit the turnover of PC. By removing the endothelium the inhitory effect is lost and the increase in turnover proceeds. Should the substance responsible for this inhibition be a water soluble product that is secreted by the endothelium, then its demonstration may be possible using the perfusion system described in this study.

3.11.1 Perfusion of control and de-endothelialized segments in series

This experiment was conducted using six segments. Two segments were de-endothelialized and kept isolated in the normal manner. An additional de-endothelialized segment was perfused in series with an intact segment joined with a short length of perfusion line. This arrangement was set up in duplicate. The results from this experiment

are presented in Table 16. The extent of ^{3}H-acetate incorporation into PC in the control segments was low and similar to that observed in previous experiments. The de-endothelialized segments whether perfused in series or in isolation all showed a three-fold increase in labelled acetate uptake relative to the controls. This procedure was carried out three times with similar results. Mean values for three experiments were 79,369 $\pm$ 12, 203 cpm/mg protein in PC compared with 66,231 $\pm$ 23,508 cpm/mg in PC for isolated segments or segments perfused in series respectively. This observation suggests that no endothelial cell derived substance mediates the increase in PC turnover apparent in de-endothelialized arteries.

Table 16. Effect of Endothelial cell conditioned perfusate on PC incorporation of ^{3}H-acetate in de-endothelialized arterial segments. 16 hour perfusion was conducted as described with the following modification. Two de-endothelialized segments were perfused in isolation in the normal fashion. A third de-endothelialized segment was placed immediately downstream from a control (with endothelium) segment which resulted in one 'in series' perfusion circuit. Two in series circuits were set up in this manner. ^{3}H-acetate was included for the last 4 hours and tissues collected, extracted and incorporation of labled acetate into PC determined as described in methods.
*Not significantly different from PC from de-endothelialized segments perfused in isolation.

Segment		Uptake of ^{3}H-acetate into PC (cpm/mg protein)
Isolated and de-endothelialized		
	1	71,390
	2	63,511
In series		
control	3	29,341
de-endothelialized	4	58,409 *
control	5	22,163
de-endothelialized	6	68,077 *

4. CONCLUSION

4.1 Introduction

Many questions remain concerning the physiology of the artery wall and disease states such as atherosclerosis. That the artery in vivo remains a particularily difficult tissue to study has prompted investigators to explore the potential of EC, SMC, platelets and macrophage cells and their interelationships in tissue culture. The activation of platelets and the release of growth factors that modify normal SMC behavior and metabolism is one example, demonstrated primarily through studies conducted in vitro (Ross et al, 1974). Incubation of LDL with arterial cells will result in a chemically modified lipoprotein that is avidly taken up by the macrophage (Henriksen et al, 1981). These observation imply that blood-borne components may also be subject to change mediated by arterial cells altering their normal function and degradation in vivo. While a great wealth of information has accumulated on the biology of blood cells, cells of the artery and the interaction with blood components such as lipoproteins, the physiological significance of many of these in vitro observations remains to be established.

An adequate explanation to account for the site-specific occurance of arterial plaques has still to be presented. In 1976, Ross and Glomsett proposed that focal 'injury' to the endothelium is a primary event in the development of lesions. Consequently, considerable work has been conducted in the intact animal. Many elaborate studies have been conducted in which regions of artery are de-endothelialized.

In the absence of endothelium a variety of observations have been made. Though exposure of the subendothelium initially promotes activation and aggregation of platelets, this process declines in a few hours. In long term studies on de-endothelialized arteries, areas of endothelial regeneration have been shown to exhibit altered permeability which coincides with subendothelial accumulation of lipid and extra-cellular matrix material such as GAGs and collagen. The demonstration that LDL may form complexes with GAGs in vitro (Iverius, 1972) and the observation of GAG accumulation in regions of endothelial regeneration suggests a mechanism whereby LDL may become trapped in the artery wall at sites of endothelial regrowth. The factors responsible for endothelial injury and the conditions in the wall of the artery that subsequently favor the development of plaque remains an area of intense investigation.

4.2 Conclusion

In the study presented here, the early effect of endothelial denudation on lipid metabolism of the artery was examined. A procedure to perfuse arterial segments in isolation was developed and provided a means to control perfusate composition while retaining the structural integrity the artery. A two step surgical method was employed to perfuse segments of rabbit carotid artery *in situ*. The segments were then isolated from the animal and maintained in an organ bath. At no time during this process were the segments permitted to de-pressurize. Those segments designated as controls (with endothelium) were shown by SEM to retain a confluent endothelial lining during the entire perfusion period. De-endothelialization of segments was carried out by advancing the supporting canulae towards each other which gave rise to endothelial loss with no apparent damage to the underlying elastic lamina as viewed under SEM.

An initial evaluation of the metabolic status of incubated arterial tissue showed that the ability to regulate *de novo* cholesterol synthesis was retained. Segments incubated in the presence of LPDS supplemented media showed an increase in ^{3}H-acetate incorporation into cholesterol when compared to similar segments incubated with FCS. The same up-regulation of cholesterol synthesis was observed in arterial segments perfused with media containing LPDS relative to their FCS perfused counterparts. These observations are consistent with *in vitro* studies on fibroblasts which also show enhanced cholesterol synthesis in the absence of LDL. Removal of LDL from the culture medium is also accompanied by a reduction in the rate of cholesterol

esterification in cultured fibroblast cells. No significant reduction in esterification of cholesterol was seen in both incubated arterial tissue or perfused segments exposed to LPDS supplemented media. To study lipid synthesis ^{3}H-acetate is often used since this precursor will label phospholipid fatty acids, and the acyl portions of DG and TG. ^{3}H-acetate will also incorporate into the cholesterol molecule. However labelled acetate is not an ideal precursor for demonstrating alterations in cholesterol esterification since both the fatty acid and cholesterol will be labelled. By removing LDL, newly synthesized cholesterol will also have a higher relative specific activity. These factors will contribute to the net radioactivity of cholesterol ester and may obscure any alteration in esterification in the absence of LDL in arterial tissue.

The elucidation of many biochemical processes has been made possible through the systematic use of radiolabelled precursors. Much of this work was conducted on isolated tissue slices and more recently in cultured cells. In these settings, problems such as isotope dilution or accessibility are minimized. However, in studies using intact perfused organs or whole animals it is important to demonstrate that radiolabelled precursors have the same degree of access in the control and experimental situation. The objective of this study was to evaluate the short-term effect of de-endothelialization on lipid metabolism in the artery wall. However the removal of endothelium may also favor increased access of ^{3}H-acetate and thus present precursor to the tissue of the artery in higher concentration than in segments with endothelium. This issue was addressed in two ways. The first was

to perfuse segments with labelled acetate added to the perfusate and monitor the appearance of radioactivity in the organ bath. The second approach was to add ^{3}H-acetate to the organ bath and measure radioactivity appearing in the perfusate. In segments lacking endothelium, a marked increase in ^{3}H-acetate uptake into PC was observed. This observation will be discussed below. In evaluating the flux of radioactivity from the perfusate to organ bath, or the reverse process, no difference was seen between segments with or without endothelium. Therefore the endothelium does not appear to represent a significant barrier for the movement of ^{3}H-acetate. Further evidence that control and de-endothelialized segments were exposed to comparable ^{3}H-acetate concentration across the arterial wall was the observation that neutral lipid and phospholipid with the exception of PC showed similar incorporation of ^{3}H-acetate in both groups. Should endothelialization permit greater access of labelled acetate, then a generalized increase in ^{3}H-acetate uptake in all lipid classes would be expected.

From this preliminary work, it was concluded that the perfusion technique described here represents a useful model for the study of artery wall metabolism in isolation. It was observed that during the surgical removal and perfusion of arterial segments, the endothelial lining remained intact as demonstrated by SEM. Segments in perfusion also retained the ability to regulate de novo cholesterol synthesis. Finally, the endothelium did not appear to present a barrier to the flux of labelled acetate in this system.

The objective of this study was to evauate the early effects of endothelial removal on lipid metabolism. The first of these experiments used MEM supplemented with 10% FCS. In de-endothelialized segments, a marked increase in the incorporation into PC was seen while the uptake of labelled acetate into the remaining polar lipids and neutral lipids parallelled that of the controls. ^{35}S-methionine uptake into tissue protein was also similar between the two groups implying no increase in protein synthesis. A similar observation was made when LPDS supplemented perfusate was used. De-endothelialized segments demonstrated a two-to-four fold increase in ^{3}H-acetate incorporation into PC while precursor uptake into the other lipid classes and protein were unchanged relative to controls. Phospholipid mass determination showed that although PC from de-endothelialized segments posessed a marked increase in radioactivity, no net increase in the amount was seen. These observations coupled with the finding that the increase in ^{3}H-acetate uptake by PC from de-endothelialized segments does not occur when serum-free perfusate is used support the conclusion that this effect requires serum but not lipoproteins to proceed. Perfusion of de-endothelialized segments in the presence of increasing concentration of LPDS showed a graded increase in the extent of ^{3}H-acetate uptake by PC and no increase in PC mass. Incorporation of ^{35}S-methionine was comparable between segments perfused with increasing LPDS concentrations. The persistent observation of enhanced uptake of labelled acetate but no increase in PC mass would suggest a process of turnover of PC in de-endothelialized segments. Also the plateau-like incorporation of labelled precursor into protein in the presence of increasing PC turnover suggests that this process is mediated by pre-existing enzyme.

The PC moiety participating in increased turnover was demonstrated in three ways. Double label experiments including ^{3}H-choline along with ^{14}C-acetate showed that greater turnover in PC of de-endothelialized segments was reflected only in the extent of [14C] detected in PC. ^{3}H-choline incorporation was similar to the controls suggesting no involvement of the choline head group of the PC molecule. By prelabelling six arterial segments (using ^{3}H-acetate) and then de-endothelializing three of the segments, a greater disappearence in ^{3}H-PC was seen in those segments without endothelium. Finally, site-specific hydrolysis of PC from control and de-endothelialized segments with PA2 showed that increased PC turnover due to endothelial loss occurs through a deacylation-reacylation process acting exclusively at the sn-2 position of the PC molecule.

The normal turnover of cellular phospholipids is a complex phenomenon. Estimates for the turnover of different phospholipids in different tissues range from a few hours to several days (Van den Bosch, 1980). This process is further complicated by possible stimulation by hormones or other factors such as platelet-derived growth factor (Leslie et al, 1982). Phospholipid turnover has been described in neuroblastoma cells (Chakravarthy et al, 1986). Differential turnover of PC head group and acyl chains was demonstrated to occur in microsomal and PM subfractions. The potential for PL modification in the PM may, in part, explain the observation of the unique PL compositions of different subcellular membranes within the cell. Phospholipid turnover has also been described in the perfused heart of rat from animals maintained on a Vitamin E deficient diet. Greater turnover was observed in PC and PE involving reacylation with

arachidonic acid occuring primarily at the sn-2 position on the phospholipid molecule. These observations suggest that Vitamin E may be important in the regulation of arachidonic acid release and eicosenoid production (Chan et al, 1985).

Studies on de-endothelialized arteries and areas of endothelial regrowth in vivo have demonstrated alterations in total phospholipid content and changes in phospholipid fatty acid species (Alavi et al, 1983). These observations have been made in long term studies. An extended time interval may be required before such changes become apparent. However, the immediate effects of endothelial loss and the mechanisms responsible for compositional changes detected some time after de-endothelialization are not well understood.

The results presented here show that in response to endothelial loss, enhanced turnover in the sn-2 fatty acyl chains of PC occurs. Deacylation or reacylation with a specific fatty acid species might provide insight into the significance of this observation. Demonstration of arachidonic acid turnover would imply involvement of eicosenoid synthesis. Changes in the degree of fatty acid unsaturation would suggest an alteration in the fluidity of PC due to endothelial loss. However no species specific shift in fatty acid composition was apparent, thus the significance of this observation is unknown.

It was demonstrated however, that the increase in PC turnover in response to de-endothelialization is mediated by a serum component though not lipoprotein. Enhanced turnover was observed in de-endothelialized arteries perfused with LPDS but not with serum-free perfusate. Preliminary evaluation of the serum factor showed that the factor promoted PC turnover in a dose-dependent fashion.

In an attempt to characterize this factor further, it was also possible to precipitate the active factor from LPDS with ammonium sulphate suggesting that it is a protein. However, when resuspended and perfused through de-endothelialized arteries, the factor no longer promoted greater PC turnover. The requirement for serum to maintain cells in culture is well known. This ability to keep cells viable is due in part to lipoproteins which provide cholesterol for cell growth and the presence of growth factors. Platelet-derived growth factor is one such factor and has been shown to have profound effects on cells in culture. These effect include increased de novo phospholipid and cholesterol synthesis and cholesterol esterification demonstrated in fibroblasts (Leslie et al, 1982). Platelet-derived growth factor also promotes an increase in LDL receptor number on SMSs (Chait et al, 1980). These observations have important implications in atherogenesis. The possibility of platelet derived growth factor mediating enhanced turnover of PC in de-endothelialized arteries described here can not be ruled out.

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